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EFFECT OF LOW TEMPERATURE ON THE TRAFFICKING OF 215 kD MANNOSE-6-PHOSPHATE/IGF-II RECEPTORS IN RAT CLONE 9 HEPATOCYTES

Beverly Jean Stoute

YALE UNIVERSITY

1990

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A Thesis Submitted to the Yale University School of Medicine
in Partial Fulfillment of the Requirements for
the Degree of Doctor of Medicine

by
Beverly Jean Stoute
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To my dear uncle, Dr. Harvey J. Whitfield, Sr., who encouraged me by his words of advice and moral support, by his example of academic excellence, and by the sensitivity and the humanity with which he regarded his patients. I regret that he did not live to see the completion of this work.

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ABSTRACT

EFFECT OF LOW TEMPERATURE ON THE TRAFFICKING OF 215 kD MANNOSE-6-PHOSPHATE/IGF- II RECEPTORS IN RAT CLONE 9 HEPATOCYTES. Beverly J. Stoute, Lisa M. Matovcik, Joseph G. Goodhouse, and Marilyn G. Farquhar. Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut.

Reduction in temperature has been used extensively as a tool for disrupting membrane and intracellular protein traffic at defined stations along both the endocytic and exocytic pathways. In the present study, the effect of low temperature on the intracellular trafficking of mannose-6-phosphate (Man6P) receptors in rat Clone 9 hepatocytes was investigated using indirect immunofluorescence (IF) and immunoperoxidase (IP) localization of receptors at the electron microscopic level. Clone 9 hepatocytes were treated with ammonium chloride (NH_4Cl) or chloroquine for 1 h, 2 h, and 3 h at 37°C and at 20°C to follow receptors as they became progressively trapped in endosomes. The percentage of cells with "Golgi," "Mixed Endosomal-Golgi," and pure "Endosomal" immunofluorescence signals was determined at each time point. The presence of immunodetectable Man6P receptor in each intracellular locale at the IF level was confirmed by diaminobenzidine localization at the electron microscopic level. To follow receptors as they recycled back to the Golgi complex, Clone 9 hepatocytes were first treated with NH_4Cl or chloroquine for 3.5 h at 37°C to trap receptors in endosomes, then incubated in weak base-free media at 37°C and at 20°C to allow receptors

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Pha[illegible] distinction [illegible] strong both [illegible] effect of [illegible] phospho[illegible] using [illegible] localization [illegible] microscopy [illegible] for [illegible] bacteria [illegible] "Color" [illegible] immunoflu[illegible] presence [illegible] at the [illegible] electron micro[illegible] the Golgi complex. Chloro[illegible] chloroquine for [illegible] incubated in weak base (raw media [illegible] to travel back to the Golgi complex. Results reported in this study demonstrate that the trafficking of Man6P receptors from the Golgi complex (receptor sorting site) to endosomes (receptor delivery site) proceeds at a slower rate, but is not disrupted at 20°C. The results obtained using NH_4Cl and chloroquine are identical except that the recycling of Man6P receptors back to the Golgi complex after chloroquine removal is slower than recycling after NH_4Cl removal. We conclude that movement of intracellular Man6P receptors from the Golgi complex to endosomes and recycling from endosomes back to the Golgi complex proceeds at 20°C, indicating that Man6P receptor traffic is not sensitive this temperature.

ABBREVIATIONS

BSA	bovine serum albumin
FBS	fetal bovine serum
IF	immunofluorescence
IGF-II	insulin-like growth factor II
IP	immunoperoxidase
Man6P	mannose-6-phosphate
MEM	Minimum essential medium
NH_4Cl	ammonium chloride
PB	phosphate buffer
PBS	phosphate buffered saline
$t_{1/2}$	half life

GENERAL INTRODUCTION

GENERAL INTRODUCTION

The targeting of intracellular proteins to their correct destinations has been the subject of central concern in recent years. Much of our knowledge has been derived from studies utilizing low temperature blocks to disrupt intracellular traffic to allow us to study discrete steps along the endocytic and exocytic pathways in a number of cell systems.

Contemporary research has shown that mannose-6-phosphate receptors function in the targeting and in the transportation of newly synthesized lysosomal enzymes; they are the only receptors for which a specific Golgi sorting signal has been identified. Specific events involved in the sorting of lysosomal enzymes have been the subject of many studies, and recently it has been demonstrated that endosomes are the delivery site of lysosomal enzymes. Further, the bulk of intracellular mannose-6-phosphate receptors was found to be trapped in endosomes after the application of weak base treatment (Brown and Farquhar, 1986; Matovcik, 1989a,b). This supported the hypothesis that the delivery site for lysosomal enzymes is an endosome. Subsequently, Woods and his coworkers took the work a step further and demonstrated that the delivery site was in fact a late endosome (Woods et al., 1989).

The purpose of the present study is to investigate the effects of low temperature on the trafficking of mannose-6-phosphate receptors between the Golgi complex (sorting site) and endosomes (delivery site).

REVIEW OF THE LITERATURE

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LYSOSOMES AND LYSOSOMAL ENZYMES

The concept of "intracellular digestion" is an old one formulated by biologists and pathologists even before the advent of sophisticated microscopes. Over a century ago, Metchnikoff spearheaded what might be called the modern era in cell biology when he evolved the revolutionary concept that phagocytic cells serve a protective function in resisting infection (Metchnikoff, 1893; Metchnikoff, 1905). This theory directly opposed accepted biological tenets of the time which postulated that phagocytic cells spread ingested organisms throughout the circulation. Metchnikoff's pioneering work on the phagocytic activity of ameboid defensive cells sparked renewed interest in the concept of intracellular digestion. Based on this work, many investigators postulated that intracellular digestion might take place in the acidic environment of some intracellular compartment.

The discovery by de Duve and his co-workers in the early 1950's of a cellular organelle termed the 'lysosome,' and its subsequent characterization as a discrete biological entity, opened an entirely new vista for biological and biochemical research (De Duve et al., 1955). Research initially focused on the biochemical isolation and characterization of the lysosome and on its unique intraorganellar hydrolytic enzymes. As our knowledge advanced, researchers became interested in the biogenesis and functional significance of lysosomes within cells. Lysosomes came to be seen as the effector organelle of all

intracellular digestion (De Duve and Wattiaux, 1966). This concept, in many ways, seems to have evolved from Metchnikoff's early work. The question of how cells sort proteins and target newly synthesized proteins to their intracellular destinations has influenced the trend of current research in all areas of cell biology. At present, there is interest in how hydrolytic enzymes are specifically sequestered within lysosomes.

The uptake of extracellular lysosomal enzymes was originally thought to take place by pinocytosis (Neufeld and Cantz, 1971). Normal fibroblasts secreted what Neufeld and her co-workers termed "corrective factors," and when these factors were added to the medium of fibroblasts from patients with I-cell disease, a syndrome characterized by the accumulation of undigested intracellular debris, they were taken up by the mutant cells (Neufeld and Cantz, 1971; Hickman and Neufeld, 1972). Later studies revealed that these "corrective factors" were lysosomal enzymes that were indeed endocytosed by a saturable and selective process (Bach et al., 1972; Neufeld et al., 1975). This implied that fibroblasts might possess cell surface receptors which facilitated lysosomal enzyme endocytosis and that these specific receptors might play a role in the transport of acid hydrolases to lysosomes (Neufeld et al., 1975).

Interest in lysosomal enzymes was intensified in the late 1970's when over thirty inherited lysosomal storage disorders were identified and described in the medical literature. (Raivio and Seegmiller, 1972; Neufeld et al., 1975; Kornfeld, 1986; Watts and Gibbs, 1986). A key development during this period was the finding that the defect in I-cell disease resulted from a deficiency in the enzyme N-acetylglucosamine-1-phosphotransferase which prevented addition of the correct phosphate address marker necessary for lysosomal enzyme targeting (Neufeld et al.,

1980; Hasilik et al., 1981; Reitman and Kornfeld, 1981a,b; Reitmen et al., 1981). There was the additional finding that alkaline phosphatase treatment of ß-glucuronidase inactivated the uptake properties of the enzyme without destroying its catalytic activity. This finding implied that the phosphate moiety must be a constituent of the enzyme's chemical structure and that it was essential for enzyme uptake (Kaplan et al., 1977a). These data led Hickman and his co-workers to postulate that many lysosomal enzymes share a common type of recognition marker which facilitates their intracellular sequestration.

In 1976, Hieber and her co-workers demonstrated that ß-galactosidase assimilation by fibroblasts was inhibited by mannose, methyl α- and ß-mannopyranosidases and mannose-containing testicular proteins; this study provided strong evidence that mannose must be a constituent of the recognition marker. Later studies revealing that mannose-6-phosphate was a competitive inhibitor of enzyme pinocytosis that was 1000-times more potent than ß-glucuronidase confirmed that hypothesis (Kaplan et al., 1977a,b). These findings suggested that the common recognition marker, required for lysosomal enzyme uptake, must contain a chemical structure related to mannose-6-phosphate, and since alkaline phosphatase treatment of fibroblasts greatly reduced uptake of ß-glucuronidase, Kaplan and his co-workers hypothesized that the phosphate moiety was present in a monoester linkage (Kaplan et al., 1977a). Investigators in the field were able to evolve a compelling hypothesis to explain the intracellular events of lysosomal enzyme traffic based on these pioneering studies. They postulated that the common recognition marker, required for the uptake of all lysosomal enzymes by specific receptors, must contain Man6P.

After newly synthesized lysosomal hydrolases are translocated across the endoplasmic reticulum membrane and the signal peptide is enzymatically cleaved, the hydrolases are post-translationally modified in the transitional elements of the endoplasmic reticulum or in the *cis* region of the Golgi complex, and receive mannose-rich, asparagine-linked oligosaccharide side chains (Li et al., 1978; Rosner et al., 1982; Sahagian, 1984). These oligosaccharide side chains are then selectively modified in the Golgi apparatus by the sequential action of the carbohydrate modifying enzymes: UDP-N-acetylglucosamine: glycoprotein N-acetyl-glucosamine-1-phosphotransferase and α-N-acetyl glucosaminyl phosphodiesterase, which specifically recognize and modify lysosomal enzymes (Rosenfeld et al., 1982). Ultimately, the transfer of α-N-acetylglucosamine 1-phosphate to mannose residues of the oligosaccharide followed by the selective removal of the N-acetylglucosamine residues leads to the overall addition of the crucial terminal mannose-6-phosphate residue on the newly synthesized protein (Reitman and Kornfeld 1981a,b; Lazzarino and Gabel, 1988; Lazzarino and Gabel, 1989). It is this mannose-6-phosphate residue that is recognized and selectively bound by mannose-6-phosphate receptors within the Golgi complex. (Neufeld and Ashwell, 1980; Sly and Fischer, 1982; Duncan and Kornfeld, 1988)

From these research beginnings, it has been established that lysosomal enzymes are removed from the secretory pathway (Ericson et al., 1984; Goldberg et al., 1984; Hasilik et al., 1984; Green et al., 1987) and delivered to their intracellular destination by virtue of a receptor-mediated process that involves interaction between phosphomannosyl residues on newly synthesized lysosomal enzymes and specific mannose-

6-phosphate (Man6P) receptors (Sly and Fischer, 1982; Creek and Sly, 1984; Kornfeld and Mellman, 1989).

MANNOSE-6-PHOSPHATE RECEPTORS: CHARACTERIZATION, DISTRIBUTION AND TRAFFICKING

Originally studied because of their role in the endocytosis of extracellular enzymes (Sando and Neufeld, 1977; Rome et al., 1979), Man6P receptors have now been shown to function in transporting newly synthesized enzymes to lysosomes (Fischer et al., 1980a,b; Gonzalez-Noriega et al., 1980; Sly and Fischer, 1982; Creek and Sly, 1984).

In 1981, Man6P receptors were isolated from bovine liver and partially characterized as monomeric glycoproteins with an apparent molecular weight of 215 kD by polyacrylamide gel electrophoresis (Sahagian et al., 1981) and later the availability of cDNA made it possible to elucidate the amino acid sequence in the protein structure (Lobel et al., 1987; Lobel et al., 1988). The discovery of cell lines which contained acid hydrolases localized to lysosomes but which were deficient in the 215 kD Man6P receptor led researchers to postulate that another mechanism for lysosomal enzyme targeting must exist (Gabel et al., 1983; Goldberg et al., 1983). This discovery led, in part, to the isolation of a second, cation-dependent 46 kD molecular weight receptor (Hoflack & Kornfeld, 1985a, b). Most cell types studied thus far appear to have both types of receptors, except for a few murine tissue culture cell lines such as P388D$_1$ macrophages (Gabel et al., 1983), and receptor-defective Chinese hamster ovary cells (Sahagian et al., 1982) which are all deficient in the 215 kD receptor. Both Man6P receptors are glycoproteins and their biology has

been recently reviewed (von Figura et al., 1986; Kornfeld and Mellman, 1989).

Characterization and Distribution of the 215 kd Man6P Receptor

The 215 kD Man6P receptor, originally purified from bovine liver (Sahagian et al., 1981; Sahagian et al., 1982; Steiner and Rome, 1982;) and from rat chondrosarcoma (Steiner and Rome, 1982), has no known ion requirement for binding and, further, binds ligand over a wider pH range than the 46 kD receptor (Tong and Kornfeld, 1988). Amino acid sequences deduced from complementary DNA revealed that the 215 kD Man6P receptor is the same protein as the IGF-II receptor and can be shown to bind IGF-II (Morgan et al. 1987; MacDonald et al., 1988); the 46 kD receptor does not bind IGF-II (Morgan et al., 1987; Tong et al., 1988; Kiess et al., 1988). The functional significance of this findings has not been established, although it suggests that the dual role of cell surface Man6P receptors may involve both lysosomal enzyme recognition and IGF-II endocytosis (Morgan et al., 1987; Waheed et al., 1988; Braulke et al., 1989).

Man6P receptors have been localized by immunocytochemistry to the cell surface (von Figura and Hasilik, 1986), to coated vesicles (Rome et al., 1979; Farquhar, 1981; Brown and Farquhar, 1984b), to endosomes (Brown and Farquhar, 1986) and to Golgi cisternae in a number of several cell types (Creek et al., 1983; von Figura et al., 1984; Braulke et al., 1987; Willingham et al., 1983; Sahagian 1983; Brown and Farquhar 1984; Geuze et al., 1984). In fact, distinct populations of coated vesicles which contain Man6P receptors have been isolated (Rome et al., 1979). The role of cell surface Man6P receptors is not fully understood (von Figura and Hasilik, 1986) although a number of studies have suggested that the cell surface pool of

Man6P receptors rapidly exchanges with the intracellular pool (Willingham et al., 1981; Merion et al., 1983). The internalization of exogenous ligand occurs via the 215 kD receptor, but there is no evidence to suggest that the 46 kD receptor can mediate enzyme internalization at the cell surface (Gartung et al., 1985; Stein et al., 1987).

The intra-Golgi localization of the 215 kD Man6P receptor has been the subject of controversy. The receptor has been localized by immunocytochemistry to the Golgi complex in several tissue and cell types (Willigham et al., 1983; Brown and Farquhar, 1984; Geuze et al., 1984) and is concentrated in the *cis* Golgi cisternae in hepatocytes, exocrine pancrease cells, and epididymmal epithelia (Brown and Farquhar, 1984a,b), across the entire Golgi stack in osteoclasts (Baron et al., 1984; Baron et al., 1985; Geuze et al., 1984; Geuze et al., 1985), and in the *trans* Golgi cisternae in ovary and other cell types (Willingham et al., 1983; Goldberg and Kornfeld, 1983; Vladutiu, 1983; Geuze et al., 1984; Geuze et al., 1985; Duncan and Kornfeld, 1988). In an effort to resolve these conflicting reports, Brown and Farquhar (1984) analyzed the distribution of the 215 kD Man6P receptor in three different cultured cell lines and compared these results to those obtained by a consistent immunocytochemical localization technique in the same cell types *in situ.* Their results suggested that there exists variability with respect to intracellular distribution among functionally distinct cell lines and tissues *in vitro* or *in vivo.* The significance of these variable findings with respect to Man6P receptor sorting is not well understood, but raises the possibility, as Brown and Farquhar (1987) postulate, that this *cis* Golgi reservoir might represent an intracellular storage compartment.

Characterization and Distribution of the 46 kD Man6P Receptor

The 46 kD receptor is present in membranes as a dimer of 46 kD subunits and, unlike its 215 kD monomeric counterpart, requires divalent cations for optimal ligand binding (Hoflack and Kornfeld 1985a,b). The 46 kD receptor binds ligands over a narrow pH range (Tong and Kornfeld, 1989) and lacks the ability to bind phosphomannosyl residues with diester linkages such as those present on *Dictyostelium discoideum* lysosomal enzymes (Freeze, 1985; Cardelli et al., 1989). The 46 kD and 215 kD receptors have sequence homology in the luminal ligand binding region (NH_2-terminal), while the transmembrane and cytoplasmic (COOH-terminal) domains are structually distinct (Von Figura et al., 1985; Dahms et al., 1987; Pohlmann et al., 1987).

The 46 kD receptor appears to function in the transportation of lysosomal enzymes in cells deficient in the 215 kD receptor. Cells which contain the 46 kD receptor in the absence of the 215 kD receptor still target lysosomal enzymes to lysosomes (Hoflack and Kornfeld, 1985b), and these mutant cells will secrete lysosomal enzymes into the extracellular medium if blocking antibodies to the 46 kD receptor are introduced into the cells (Gartung et al., 1985; Stein et al., 1987). It is unclear, however, if the function and kinetics of the 46 kD receptor is directly analogous to the 215 kD receptor since the 46 kD Man6P receptor has not been as extensively studied; a few recent studies have provided compelling preliminary data on this point.

Biochemical studies have revealed that both the 215 kD and the 46 kD receptors are found in similar membrane fractions in bovine liver (Duncan and Kornfeld, 1988) and that the 46 kD receptor travels through the *trans* Golgi complex in Chinese hamster ovary cells (Duncan and Kornfeld, 1988).

To date only two studies have been published which investigated the distribution of the 46 kD receptor using immunocytochemical techniques. The first study reported that the 46 kD receptor can be localized *in situ* to tubules and small vesicles adjacent to the *trans* Golgi region (Bleekemolen et al., 1988) in U937 monocytes. The second study demonstrated that the intracellular distributions of the 46 kD and the 215 kD Man6P receptors partially overlap, and that the 46 kD Man6P receptor transports lysosomal enzymes to a destination which seems to be identical to the late endosomal delivery site of the 215 kD Man6P receptor (Matovcik et al., 1989). The subject of the 46 kD Man6P receptor function and kinetics will be, undoubtedly, a subject of intense future investigation.

215 kD Mannose-6-Phosphate Receptor Trafficking:

Newly synthesized secretory proteins traverse a common pathway from their site of synthesis in the rough endoplasmic reticulum through the terminal compartment of the Golgi complex. It is believed that sorting of newly synthesized proteins takes place in the terminal region of the Golgi complex, or the *trans* Golgi network (TGN), before their targeting to distinct post-Golgi destinations (Palade, 1975; Farquhar, 1985; Griffiths and Simons, 1986). Cycling of Man6P receptors between the Golgi and endosomes is accomplished via clathrin-coated vesicles (Friend and Farquhar, 1967; Campbell et al., 1983; Campbell and Rome, 1983; Lemansky et al., 1987; Brown and Farquhar, 1984b). After enzyme-receptor complexes exit the Golgi complex, it has been postulated that the Golgi-derived vesicles fuse with an acidic prelysosomal compartment (Griffiths et al., 1988) recently characterized as a late endosome (Brown and Farquhar, 1986; Woods et al., 1989) where ligand dissociation is triggered

in the low pH environment. Researchers hypothesize that the lysosomal hydrolases are then routed to lysosomes, while the Man6P receptors are directed back to the Golgi complex-- never reaching the lysosomal compartment (von Figura et al., 1984; von Figura and Hasilik, 1986; Woods et al., 1989).

The kinetics and turnover of the 215 kD Man6P receptor have been extensively studied (Creek and Sly, 1983b; Goldberg et al., 1983; Sahagian et al., 1984; Brown and Farquhar, 1986). The receptor $t_{1/2}$ is 20-24 hours in Clone 9 hepatocytes (Brown and Farquhar, 1984) with a range of 6-32 hours in other cell types (Sahagian and Neufeld, 1983; Creek and Sly, 1983b). The time required for a round trip between the Golgi and endosomes, according to the work of Brown et al. (1986) in Clone 9 hepatocytes, is ~85 minutes. This implies that the 215 kD Man6P receptors recycle, completing up to 18-20 round trips during their lifetime. It has been shown also that it takes Man6P receptors longer to travel from the Golgi complex to endosomes ($t_{1/2}$ ~75 min) than to recycle on the return trip from endosomes back to the Golgi complex ($t_{1/2}$~10 min) (Brown and Farquhar, 1986).

Lysosomal enzymes are the only known biosynthetic product for which a Golgi sorting signal and receptor have been identified. Little is known, however, about how lysosomal enzymes are sequestered within these transport vesicles, how transport vesicles form, or even how endosomes evolve into secondary lysosomes after they receive their pioneering population of enzymes. Perhaps these are the questions that will challenge future researchers in the field. Our knowledge of mannose-6-phosphate receptors and lysosomal enzyme sorting has increased considerably, but many questions remain unanswered. The present study

proposes to determine whether the traffic of Man6P receptors between the sorting site (Golgi) and the delivery site (endosomes) can be manipulated differentially by changes in temperature.

EFFECTS OF TEMPERATURE ON INTRACELLULAR PROTEIN TRAFFIC

The effect of temperature on many biological systems has been the subject of extensive investigation. Reduction in temperature has been used extensively as a tool for arresting membrane transport at defined stations along both the endocytic and exocytic pathways (reviewed in Mellman et al., 1986; Griffiths & Simons, 1986; Green et al., 1987; Pfeffer and Rothman, 1987). More recently, temperature-sensitive steps along both the endocytic and exocytic intracellular pathways have been identified in mammalian cells.

Ligands bind to receptors on the plasma membrane at low temperature (< 10°C) but are not internalized (Willlingham et al., 1981). At temperatures between 15°C and 20°C, internalization of cell-surface bound ligands occurs, but the internalized ligands are not delivered to lysosomes and, therefore, accumulate in prelysosomal compartments (Sandwig and Olsnes, 1979; Dunn et al., 1980; Marsh et al., 1983; Miller et al., 1986). This implies that the late fusion events in the endocytic pathway are inhibited at temperatures below 20°C (Edelson and Cohn, 1978; Dunn et al., 1980; Kielian and Cohn, 1980; Salzman and Maxfield, 1989). Even though the fusion of endosomes and lysosomes is inhibited at temperatures below 20°C, there is evidence to suggest that recycling of receptors to the plasma membrane can continue at this reduced temperature (Gabel & Foster, 1987).

More recent studies have revealed that the intracellular steps along the exocytic pathway in the transport of viral membrane proteins and secretory proteins are also differentially sensitive to temperature. A number of studies have examined intracellular protein traffic along the Golgi to plasmalemma pathway. *In vitro* studies investigating the effect of low temperature on the transport of membrane glycoproteins have provided evidence to show that newly synthesized glycoproteins exit the rough endoplasmic reticulum at 15°C but do not travel beyond an intermediate compartment between the endoplasmic reticulum and the Golgi complex (Saraste and Hedman, 1983; Saraste and Kuismanen, 1984; Matlin and Simons, 1983).

Semliki forest viral membrane proteins in infected BHK-21 fibroblasts were blocked in their transit from the *trans* Golgi cisternae to the cell surface at 15°C and, therefore, become trapped in an intermediate compartment between the endoplasmic reticulum and the Golgi (Saraste et al., 1984). Using a sensitive tryptic assay to detect the appearance of the membrane glycoprotein hemagglutinin on the influenza-virus infected Madin-Darby canine kidney cell surface, Matlin and Simons (1983) demonstrated that reduced temperature (20°C) specifically and reversibly blocks hemagglutinin externalization, although it has no effect on terminal glycosylation. Similarly, it has been shown that nucleoside membrane transport in macrophages is affected at temperatures below 25°C (Berlin, 1973), that histamine secretion from mast cells was reduced at temperatures below 16°C (Lagunoff & Wan, 1974) and that acetylcholinesterase secretion from cultures of chicken embryo muscle cells ceased abruptly at 20°C (Rotundo & Fambrough 1980).

Saraste and his co-workers (1986) took advantage of these findings to investigate in detail the effect of low temperature on the transport of secretory proteins in exocrine pancreas lobules. They demonstrated that there are distinct temperature-sensitive transport steps along the endoplasmic reticulum-plasmalemma pathway. The results they obtained from cell fractionation studies, as well as from immunocytochemical studies at the electron microscopic level, demonstrated that an advancing front of newly synthesized secretory proteins from the endoplasmic reticulum in exocrine pancreas cells reached the *cis* Golgi elements at 16°C, moved on to the *middle* Golgi compartment at 20°C, but did not advance further to reach condensing vacuoles or zymogen granules until the cells were warmed to 37°C. Thus, they defined successive transport steps of vesicular elements along the endoplasmic reticulum-plasmalemma pathway according to their individual sensitivities to temperature.

Taken together, these findings suggest that endocytosis takes place at temperatures between 15°C and 20°C, but later steps in the endocytic pathway after internalization of cell surface receptor-ligand complexes and steps along the exocytic route have specific, higher temperature requirements.

The accumulation of large quantities of proteins in specific intracellular compartments after lowering temperature is an interesting and potentially useful experimental tool. Altering intracellular protein traffic patterns at low temperature could facilitate the identification and further study of specific compartments which previously could be assessed only by kinetic studies. The temperature block, therefore, is a potentially powerful experimental tool for investigating the transport

pathway of newly synthesized proteins from the endoplasmic reticulum to the Golgi complex and from the Golgi complex to the plasma membrane.

THE EFFECT OF LYSOSOMOTROPIC WEAK BASES ON MANNOSE-6-PHOSPHATE RECEPTOR RECYCLING

Treatment of Clone 9 hepatocytes with lysosomotropic weak bases such as NH_4Cl and chloroquine leads to the elevation of intra-endosomal pH and therefore to the accumulation of receptors at their delivery site, namely late endosomes (Brown and Farquhar, 1986; Woods et al., 1989). These lysosomotropic agents elevate the pH of intracellular compartments such as endosomes and lysosomes to pH levels above 6.8 and have been used extensively for manipulating intracellular receptor traffic (Ohkuma and Poole, 1978; Gonzalez-Noriega et al., 1980; Ohkuma and Poole, 1981; Poole and Ohkuma, 1981; De Duve, 1983; De Duve et al., 1984; Brown et al., 1984; Brown and Farquhar, 1986; Gonzalez-Noriega et al., 1989). As a result of the increase in intra-endosomal pH (Reijngoud et al., 1976) lysosomal enzymes cannot dissociate from Man6P receptors and Man6P receptor-lysosomal enzyme complexes become trapped in endosomes and the receptors cannot recycle back to the Golgi complex (Brown and Farquhar, 1986). The osmotic uptake of water accompanies weak base trapping and leads to the progressive vacuolation of endosomes as the trapping of receptor-ligand-complexes takes place (De Duve, 1983; Ohkuma and Poole, 1981; Brown and Farquhar, 1986).

The acidic intra-endosomal pH is re-established after removal of the weak base, thereby facilitating dissociation of receptor-ligand complexes and allowing recycling of Man6P receptors back to the Golgi complex.

Thus, Man6P receptors can be synchronized at the sorting site (Golgi complex) or delivery site (endosomes) before experimental manipulation. In this study lysosomotropic weak bases were a valuable tool for synchronizing Man6P receptor traffic.

PURPOSE

PURPOSE

Based on previous work examining the effect of low temperature on endocytic and. exocytic pathways, the current study was designed to examine the effect of temperature manipulation on the trafficking of Man6P receptors. This study was the first to investigate the effect of low temperature on intracellular Man6P receptor traffic. The central purpose was to investigate the effects of low temperature on Man6P receptor trafficking between the Golgi complex and endosomes. Outbound traffic, that is to say, Golgi to endosomes, as well as inbound traffic, endosomes to Golgi, were investigated individually. The experimental question addressed in this connection: is the trafficking of Man6P receptors inhibited at 20°C?

Clone 9 hepatocytes were selected because they are a model system in which the traffic of the 215 kD Man6P receptor between the Golgi complex and endosomes has been well defined and can be easily synchronized using the lysosomotropic agents, ammonium chloride and chloroquine (Brown and Farquhar, 1986). Ammonium chloride and chloroquine were valuable experimental tools because of their roles in trapping Man6P receptors in endosomes by increasing intra-endosomal pH. Since previous studies reported that a number of endocytic and exocytic events are interrupted at 20°C, this temperature was selected for study.

MATERIALS AND METHODS

MATERIALS AND METHODS

Materials:

Ammonium chloride, diaminobenzidine hydrochloride (type II), saponin, bovine serum albumin, chloroquine, L-lysine monohydrochloride, potassium ferrocyanide, p-phenylenediamine and sodium m-periodate were purchased from Sigma Chemical Company (St. Louis, MO). Minimal essential medium (MEM) (Earle's salts), penicillin-streptomycin, and fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY). Rhodamine-conjugated goat anti-rabbit IgG was purchased from Tago (Burlingame, CA) and sheep anti-rabbit IgG conjugated to horseradish peroxidase (HRP) were obtained from Biosys (Compiegne, France). Sodium phosphate, monobasic and dibasic were purchaed from Baker (Phillipsburg, NJ); EPON 812, lead citrate and cacodylic acid (sodium salt) were purchased from Ernest F. Fullam, Inc. (Schenectady, NY). Glutaraldehyde and osmium tetroxide were purchased from Electron Microscopy Sciences (Fort Washington, PA). Tris hydrochloride was purchased from Schwartz/Mann Biotech (Cleveland, OH).

Cell Culture:

Clone 9 rat hepatocytes, originally derived from normal rat liver, were obtained from Dr. David Sabatini of New York University and maintained as subconfluent monolayers in freshly prepared MEM (Earle's

salts) containing 10% heat inactivated FBS, 100 U/ml penicillin G, and 100µg/ml streptomycin. Cells were subcultured at 37°C for two days prior to an experiment on glass coverslips for immunofluorescence (IF) or 35 mm plastic tissue culture dishes for immunoperoxidase (IP) localization in an atmosphere of 95% air and 5% CO_2.

Antibody:

The techniques employed to prepare, characterize and purify the rabbit polyclonal anti-Man6P receptor IgG have been previously described (Brown and Farquhar, 1984). Man6P receptors were isolated from Triton X-100 extracts prepared from rat liver membranes by affinity chromatography on a column of yeast pentamannosyl-6-phosphate oligosaccharides. A single protein of ~215 kD molecular weight was then identified (Sahagian et al.,1981) and eluted with mannose-6-phosphate, purified and used as the immunogen to generate antibodies in rabbits.

The rabbit polyclonal anti-Man6P receptor IgG antibodies recognize only the 215 kD Man6P receptor by immunoprecipitation (Brown and Farquhar, 1984.)

Incubation with Ammonium Chloride or Chloroquine:

Clone 9 cells were incubated in NH_4Cl for various time periods at 37°C. Ammonium chloride treatment elevates the intracellular pH (Gonzalez-Noriega et al., 1980; Reijgoud et al., 1976; De Duve, 1974) and causes swelling and vacuolation of endosomes and lysosomes (Ohkuma & Poole, 1978; Poole and Ohkuma, 1981; Maxfield, 1982; Galloway et al., 1983). This treatment leads to a depletion of Man6P receptors from their sorting site, namely the Golgi complex, and an accumulation in large,

dilated endosomes (Brown and Farquhar, 1986). For light microscopy, cells were treated with 30 mM NH_4Cl. For electron microscopy, cells were treated with 10 mM NH_4Cl, which resulted in smaller vacuoles but preserved the morphology of the cells. After treatment with NH_4Cl, cells were fixed for immunocytochemistry.

Clone 9 cells were incubated in MEM containing chloroquine for various time periods. Chloroquine treatment was done at a concentration of 25 µM for both light and electron microscopy. After treatment with chloroquine, cells were fixed for immunocytochemistry.

Recovery Experiments:

To examine the traffic of Man6P receptors from the endosomes to the Golgi complex, Clone 9 cells were first treated with NH_4Cl for 3.5 or 4 hours at 37°C, since virtually all intracellular Man6P receptors become associated with endosomes under these conditions (Brown anf Farquhar, 1986). Cells were washed several times in MEM precooled to 20°C and then incubated in NH_4Cl-free MEM for 30, 60 or 120 minutes at 20°C or 37°C to follow the receptor traffic from endosomes to the Golgi. Parallel experiments were done in which cells were treated with chloroquine. Since the washout of the lipophilic base, chloroquine, is more difficult than that of NH_4Cl, cells in these experiments were washed in chloroquine-free MEM, incubated 10 minutes in chloroquine-free MEM, and then washed again with chloroquine-free MEM before the end of the incubation period to ensure complete washout of the lipophilic base. During this "washout" or "recovery" period, as the weak base is removed, the acidic pH of endosomes and lysosomes is re-established allowing the return of receptors from endosomes to the Golgi complex (Ohkuma and

Poole, 1978; Maxfield, 1982; Galloway et al., 1983; Brown and Farquhar, 1986). Cells were then fixed and processed for immunofluorescence (IF) or immunoperoxidase (IP) as indicated. Cycloheximide at a final concentration of 2 mg/ml was added to the washout media to prevent new receptor synthesis.

Immunofluorescence Staining:

Following treatment, Clone 9 cells were fixed on coverslips in 2% formalin in 75 mM phosphate buffer for 45 minutes at room temperature, washed in 75 mM phosphate buffer and permeabilized with 0.05% saponin in phosphate buffered saline (PBS) containing 0.1% BSA. Cells were then incubated for 1 hour at room temperature with anti-Man6P-receptor IgG at a concentration of 6.6 µg/ml. After the initial anti-Man6P receptor incubation, cells were washed in PBS containing 0.1% BSA and incubated at room temperature with rhodamine-conjugated goat anti-rabbit IgG (10 µg/ml) for 1 hour as previously described (Brown and Farquhar, 1986)

Coverslips were then washed in PBS containing 0.1% BSA, individually mounted in p-phenylenediamine after fixation to reduce fading (Johnson and Araujo, 1981), and photographed on a Zeiss Photomicroscope III.

Immunoperoxidase Staining:

After 10 mM NH_4Cl or 25 µM chloroquine treatment, and washout of cells, periodate-lysine-paraformaldehyde fixation was employed for 4 hours at room temperature as previously described (McClean and Nakane, 1974; Brown and Farquhar, 1984a,b; Brown and Farquhar, 1986). Cells were washed in 0.1 M sodium phosphate buffer to remove fixative and then

with 0.1M sodium phosphate buffer containing 0.1% BSA (PB/BSA). Next, cells were pemeabilized in 0.005% saponin in PB/BSA for 15 minutes and then labeled at room temperature for 14 hours with anti-Man6P receptor IgG in PB/BSA containing 0.04% sodium azide. In order to prevent the reversal of the saponin permeabilization during the antibody incubations and the washes, saponin was maintained in the phosphate wash buffer at a final concentration of 0.005%. After the cells were washed in PB/BSA containing 0.005% saponin, they were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG, and then fixed in 4% glutaraldehyde in 100 mM sodium cacodylate (pH 7.4) containing 7.5% sucrose for 1 hour at room temperature.

Peroxidase reaction product was developed by incubating cells in diaminobenzidine medium (0.2% diaminobenzidine hydrochloride) with 0.01% H_2O_2. The peroxidase reaction was stopped by washing cells in 50 mM Tris HCl (pH 7.4) containing 7.5% sucrose. Cells were scraped from petri dishes in Tris-sucrose buffer, centrifuged in a Beckman Microfuge 152 for 10 minutes, and postfixed for 1 hour with 1% reduced OsO_4 (with 1% potassium ferrocyanide) in 100 mM sodium cacodylate. Postfixed cells were then washed in sucrose-free 100 mM sodium cacodylate buffer and dehydrated in graded series of ethanol (70%, 95% and 100%). After incubation at room temperature in 100% ethanol for 1 hour, cell pellets were transferred to a solution of 50% EPON and 50% ethanol and then embedded in EPON 812. Thick sections (0.5 µm) were prepared on a Reichert Jung Ultracut microtome and examined by light microscopy. Ultrathin sections (60-65 nm) were prepared with a Diatome Diamond knife and placed on formvar and carbon-coated electron microscope grids. Next, these ultrathin sections were stained with lead citrate for 2

minutes and examined on a Philips 301 electron microscope at 60 kV. This procedure has been described previously by Brown and Farquhar, 1986.

Quantification of Man6P Receptor Distribution:

In immunofluorescence experiments, each cell was classified according to location of the immunofluorescence antibody. The number of cells in which receptors were concentrated in the juxtanuclear, or Golgi region versus those cells in which receptors were concentrated in large, dilated vacuoles (endosomes), were counted at various times after cells were treated with weak base and again after removal of the base and incubation in base-free media for various time periods. Cells were scored as having a "Golgi," "Endosomal," or "Mixed" -type staining pattern in IF preparations stained with antibodies to Man6P receptors as depicted in the standard IF patterns in figures 1, 3 and 4.

When a cell was scored as "Endosomal" this implied that the immunofluorescent staining pattern consisted primarily of large dilated vacuoles without concomitant Golgi signal (Fig. 2). When a cell was scored as "Golgi," this indicated a juxtanuclear staining pattern characteristic of Man6P receptors localized to the Golgi complex (Fig 1). Lastly, when a cell was scored as "Mixed," this implied the simultaneous presence in the same cell of receptors in dilated vacuoles (endosomes) and in the juxtanuclear region (Golgi complex); see figure 4.

At least 250 cells were scored at each time point in each separate experiment. Raw scores from separate experiments were averaged and standard deviations calculated.

RESULTS

RESULTS

Steady State Distribution of Mannose-6-Phosphate Receptors in Rat Clone 9 Hepatocytes is Identical at 37°C and 20°C

Previous work has shown that the 215 kD Man6P receptors in control (untreated) Clone 9 hepatocytes are typically localized to the juxtanuclear Golgi region by IF. The presence of Man6P receptors in one or two Golgi cisternae, in Golgi-associated vesicles, or occasionally in larger vacuoles (endosomes) is responsible for this pattern (Brown and Farquhar, 1984). Clone 9 hepatocytes examined at steady state in this study show the same pattern of distribution (Fig. 1).

To examine the steady state distribution of Man6P receptors in Clone 9 cells at 20°C, untreated Clone 9 hepatocytes were washed in MEM precooled to 20°C and then incubated at 20°C for 3.5 h before fixation for IF or IP. The steady state distribution of Man6P receptors at 20°C is indistinguishable at both the IF (Fig. 1) and IP (Fig. 2) levels from the steady state distribution at 37°C.

Mannose-6-Phosphate Receptors Accumulate in Endosomes after Lysosomotropic Weak Base Treatment at 20°C

Immunofluorescence:

As shown previously, treatment of Clone 9 hepatocytes for 1 h, 2 h, and 3.5 h with NH_4Cl at 37°C results in the gradual accumulation of Man6P

receptors in large vacuoles previously identified as late endosomes using a double-labeling IF technique (Brown and Farquhar, 1986; Woods et al., 1989). As Clone 9 cells were treated with weak base for increasing periods of time, the distribution of Man6P receptors gradually changed from a predominately "Golgi" pattern to a "Mixed" pattern, and then to an "Endosomal" IF pattern in keeping with these previous results. After 3.5 h of treatment with either NH_4Cl or chloroquine at 37°C, the data presented here demonstrate that the distribution of Man6P receptors is similar to that observed after treatment at 37°C. It is clear, however, that vacuoles (endosomes) seen on phase contrast microscopic examination do not become as dilated when Clone 9 cells are treated with chloroquine or NH_4Cl at 20°C as they do at 37°C (Fig. 3).

After 1 h of NH_4Cl treatment at 37°C, 31% of cells showed the "Endosomal" IF pattern; whereas, after 2 h of treatment, 56% of cells showed an "Endosomal" pattern (Table I). After 3.5 h of NH_4Cl treatment, almost all detectable Man6P receptors, i.e. 93% of cells, showed receptors associated soley with dilated endosomes, as evidenced by the "Endosomal" IF signal; and the remaining 7% had a "Mixed Golgi-Endosome" signal.

Treatment of Clone 9 cells with NH_4Cl at 20°C also results in the accumulation of Man6P receptors in endosomes, but at slower rate than at 37°C (Fig. 4). According to the data compiled in Table 1, after 1 h of NH_4Cl treatment 84% of the cells treated at 20°C had a "Mixed" signal, 16% still had a pure "Golgi" signal, and no cells yet had a pure "Endosomal" signal. After 2 h of NH_4Cl treatment at 20°C, 29% of cells had an "Endosomal" signal and the remaining 71% a "Mixed" signal. However, the number of cells with a pure "Endosomal" signal by the end of the 3.5 h treatment period at 20°C (96%) was similar to the percentage of cells with an

"Endosomal" signal after the 37°C treatment (93%) for the same time period. This suggests, therefore, that incubation of cells at the lower temperature slows down but does not stop movement of Man6P receptors from the Golgi complex to endosomes.

Analogous experiments were done treating Clone 9 cells for 1 h, 2 h, and 3.5 h with chloroquine at 37°C and at 20°C. After treating cells for 1 h with chloroquine at 37°C, 62% of the cells showed an "Endosomal" IF staining pattern, 38% showed a "Mixed" pattern (Table II). After a 2 h treatment period at 37°C, 93% of cells had an "Endosomal" signal and 7% of cells had a persistent "Mixed" signal. Nearly all cells (i.e. 96%) showed an "Endosomal" IF pattern after 3.5 h of treatment.

Experiments conducted in which Clone 9 cells were treated with chloroquine at 20°C show the same overall results as in the case of NH_4Cl; that is, Man6P receptors do relocate from the Golgi complex to endosomes even at 20°C (Table II). 26% of Clone 9 cells still had a "Golgi" IF pattern after a 1 h chloroquine treatment, while 74% had a "Mixed" IF pattern, indicating that Man6P receptors had indeed reached the endosomes albeit more slowly. No cells showed evidence of a pure "Endosomal" IF signal after only 1 h treatment at 20°C. After 2 h of chloroquine treatment at 20°C, 74% of Clone 9 cells had a pure "Endosomal" pattern while 26% still had a "Mixed" IF pattern. Finally, after 3.5 h of treatment with chloroquine at 20°C, there was no evidence of a residual pure "Golgi" signal. In fact, 79% of cells had a pure "Endosomal" pattern and only 21% had a residual "Mixed" IF pattern.

Immunoperoxidase:

Electron microscopy confirmed that Man6P receptors were localized to endosomes after NH_4Cl and chloroquine treatment (Fig. 5). When Clone 9 cells were treated with NH_4Cl and chloroquine for 3.5 h in parallel experiments and then stained for Man6P receptors by IP, the diaminobenzidine reaction product was regularly detected in dilated vacuoles or endosomes at both 37°C and at 20°C; the results were the same for both weak bases.

Mannose-6-Phosphate Receptor Recycling from Endosomes to the Golgi Complex is Slower at 20°C than at 37°C

Immunofluorescence:

Clone 9 hepatocytes were treated with NH_4Cl for 3.5 h at 37°C to load Man6P receptors into large dilated endosomes detectable by IF. When cells were transferred to fresh (NH_4Cl-free or chloroquine-free) media and incubated over a 1-2 h period at 37°C, the size of endosomes progressively decreased and the receptors returned to the Golgi complex during the washout period (Brown and Farquhar; 1986). Since washout of weak base occurs at 37°C, Man6P receptors recycle from endosomes back to the Golgi complex.

The data compiled in table III indicate that the recycling of Man6P receptors seems to proceed at 20°C, but the rate is slower. After a 30 min washout period at 37°C, 82% of Clone 9 cells already showed a "Golgi" IF signal, and 18% of Clone 9 cells still had a "Mixed Endosomal-Golgi" IF pattern. After a 30 min washout period at 20°C, only 27% showed the same "Golgi" signal, while 73% still had a "Mixed Endosomal-Golgi" signal. Although these data imply that Man6P receptors can recycle from

endosomes back to the Golgi complex at 20°C as early as 30 min after returning the cells to lysosomotropic base-free media, it is clear that a much smaller percentage of cells manifest a pure "Golgi" signal at the lower temperature for that period of time.

Similarly, after a 1 h-washout period at 37°C 95% of cells showed a pure "Golgi" signal and 5% showed a "Mixed Endosomal-Golgi" signal. At 20°C, only 28% had a pure "Golgi" IF signal and the remaining 72% of the cells still had a "Mixed Endosomal-Golgi" signal.

After a 2 h-washout period at 37°C, nearly all Man6P receptors in Clone 9 cells recycle back to the Golgi complex. The size of phase-lucent endosomes progressively decreased during the washout period and by the the 2 h period the pattern of "Golgi" IF staining in the cells was indistinguishable from controls (Fig. 6). 99% of cells showed a pure "Golgi" IF signal; the remaining 1% had a "Mixed Endosome-Golgi" pattern. These data are consistent with the original study reported by Brown and Farquhar (1986) using NH_4Cl as a tool for investigating Man6P receptor traffic at 37°C.

In comparison, after a 2h-washout period at 20°C, 71% had a pure "Golgi" pattern while 29% of cells still had a "Mixed" signal. This implies that recycling of Man6P receptors does occur at the lower temperature but that receptors cannot be completely cleared from endosomes even after 2 h of washout.

Although the washout of chloroquine requires longer time periods to achieve than that of NH_4Cl (Brown and Farquhar, 1986), results comparable to NH_4Cl were obtained with respect to the effect of low temperature on the recycling of Man6P receptors from endosomes to the Golgi complex. After Clone 9 cells were treated with chloroquine for 3.5 h, cells were

incubated in chloroquine-free medium for 1 h and 2 h time periods (Fig. 7). As indicated by the data in table IV, after 1 h of washout at 37°C, 83% of cells had a persistent "Endosomal" pattern while 17% had a "Mixed Golgi-Endosomal" IF staining pattern. After 2 h of washout at 37°C, no cells had a pure "Endosomal" staining pattern since 68% had a "Mixed" pattern and 32% had a pure "Golgi" staining pattern.

After a 1 h washout period at 20°C, 8% of cells had a "Mixed Endosomal" pattern and 87% maintained the pure "Endosomal" IF pattern. After a 2 h washout period at 20°C, 71% of cells had an "Endosomal" pattern while 29% had a "Mixed Golgi-Endosomal" pattern, indicating that the Man6P receptor leaves the endosomal compartment more slowly at the lower temperature.

Immunoperoxidase:

These findings at the IF light microscopic level were confirmed by parallel IP experiments localizing intracellular Man6P receptors at the electron microscopic level at 37°C and at 20°C. The results obtained with NH_4Cl and chloroquine were the same except that washout of NH_4Cl from cells was faster than that of chloroquine (Figs. 8, 9, 10, 11).

After recovering from chloroquine treatment for 30 min at 37°C (Fig. 8A) or 1 h (Fig 9A), most cells still had a "Mixed" IF pattern indicating that receptors had already started to recycle back to the Golgi complex. A "Mixed" IF pattern corresponded, at the IP level, to the simultaneous finding of dilated endosomes richly stained with diaminobenzidine reaction product with peroxidase-stained Golgi cisternae within the same cell. By the end of the 2 h washout period the majority of cells still had the "Mixed" staining configuration (Fig. 10A), although a number of cells emerged with

strongly reactive Golgi cisternae in the absence of dilated endosomes, indicating that Man6P receptors had exited the endosomes and finally recycled back to the Golgi complex. In the case of NH_4Cl, most cells had pure "Golgi" IF signal (Table II) by the end of the 2 h washout period at 37°C. This finding was confirmed by IP localization of Man6P receptors in richly stained Golgi cisternae in the absence of detectable endosomal staining (Fig. 11B).

With respect to temperature, the findings with NH_4Cl and chloroquine were similar; therefore, electron micrographs for chloroquine comparing 37°C washout with 20°C washout are shown as representative of both weak bases. After the 30 min (Fig. 9B) and 1 h (Fig. 10B) time points at 20°C, it is clear that the predominant finding is that of a "Mixed" reaction product. Although one sees the receptor in the Golgi cisternae consistently and not in dilated endosomes after 2 h of washout at 37°C, the signal is still primarily a "Mixed" one after 2 h of washout at at 20°C (Fig. 10B).

In summary, electron microscopy confirmed the IF data which showed that washout of weak base led to the recycling of Man6P receptors from endosomes to the Golgi cisternae where IP reaction product was detected and, further, that the "Mixed" IF signal seemed to represent at the IP level a mixture of Golgi complex and endosomal staining. Results with NH_4Cl and chloroquine were comparable. One can unequivocally conclude that recycling of Man6P receptors from endosomes to the Golgi complex occurs at 20°C, but that the movement of Man6P receptors was indeed slower at the lower temperature, as evidenced by the finding that many cells still had large dilated endosomes filled with diaminobenzidine reaction product after 1 h (Fig.9) and even after 2 h of washout at 20°C (Fig.10).

DISCUSSION

The transport of lysosomal enzymes from their site of synthesis to their functional destination is a multistep process. Much of our knowledge about endocytic and exocytic pathways has been derived from studies utilizing low temperature blocks to disrupt intracellular transport and cause the accumulation of ligands at various stations along the intracellular route. In this thesis the main objective was to determine if low temperature could be used as a tool to dissect the component events along the transport route of Man6P receptors at various points and allow us to develop a method of manipulating intracellular traffic that will prove useful for future study.

A number of late endocytic and exocytic events are disrupted at 20°C. Transport of newly synthesized secretory proteins to zymogen granules in pancreatic exocrine cells (Tartakoff, 1985; Saraste et al., 1986), transport of secretory (Rotundo and Fambrough, 1980) and membrane proteins to the cell surface in other cell types (Matlin and Simons, 1983; Saraste and Kuismanen, 1984) are processes inhibited at temperatures below 20°C. This temperature was, therefore, chosen for study. In fact, the pivotal study conducted by Saraste and his co-workers (1986) showed that transport of newly synthesized secretory proteins from zymogen granules to the cell surface is inhibited at temperatures below 20°C. In light of this finding, it was hypothesized here that the trafficking of the Man6P receptor in the inbound direction (i.e. from endosomes to the Golgi complex) is also inhibited at 20°C.

The main findings reported here from the immunofluorescent localization of Man6P receptors and from immunoelectron microscopic studies in rat Clone 9 hepatocytes are: (1) Man6P receptor traffic in both

the inbound (endosomes to Golgi) and the outbound (Golgi to endosomes) directions proceeds at 20°C; (2) the traffic of Man6P receptors proceeds at a slower rate at 20°C than at 37°C as evidenced by the slower rate of accumulation of Man6P receptors in corresponding intracellular locales.

Man6P receptors pick up their cargo of newly synthesized lysosomal enzymes in the Golgi complex and shuttle them to endosomes via coated vesicles. The acidic pH of endosomes triggers the dissociation of the enzymes from the Man6P receptors, and the receptors return to the Golgi complex while lysosomal enzymes continue along the pathway until they reach their final destination, namely lysosomes. Weak bases are used experimentally to trap receptors along with their ligands in lysosomes or endosomes. In this study, the traffic of Man6P receptors in the outbound direction, from the Golgi complex to endosomes, was studied by treating Clone 9 cells for increasing periods of time with ammonium chloride or chloroquine.

Treatment of Clone 9 hepatocytes for up to 3.5 h leads to the progressive accumulation of Man6P receptors in endosomes (Table II) The percentage of cells with an "Endosomal" IF signal increased over 3.5 h of treatment with NH_4Cl at 37°C and at 20°C. There were no cells which had a pure "Endosomal" IF signal early in the NH_4Cl treatment intervals at 20°C, a treatment period after which labeled endosomes were commonly observed at 37°C. By the end of the 3.5 h treatment period with NH_4Cl at 20°C, however, nearly all of the cells had an "Endosomal" IF signal, indicating that movement of the Man6P receptors from the Golgi complex to endosomes was indeed complete and, further, that virtually all immunodetectable Man6P receptors were localized to large dilated

endosomes after treatment at 37°C and at 20°C. Comparable results were obtained using chloroquine (Table II).

It is also noted that the size of vacuoles or endosomes visualized by IF and phase contrast microscopy is not as large as at 20°C as at 37°C. The pH raising effect of lysosomotropic agents is postulated to result in the osmotic uptake of water which leads to vacuolation of specific intracellular compartments, namely lysosomes or, in this case, endosomes (De Duve, 1983; Ohkuma and Poole, 1981). As lysosomes increase in volume, especially as the osmotic uptake of water accompanies weak base trapping, membrane reorganization presumably takes place involving the fusion of many small lysosomes to form larger vacuoles or the recruitment of membrane from some other source. According to the work of Poole and Ohkuma (1981), the second phase in the uptake of weak bases into lysosomes involves the pumping of protons into the organelle in order to maintain the pH inside the lysosomes at a steady, elevated value. Previous studies have demonstrated that the process of proton accumulation in lysosomes is energy-dependent, since metabolic poisons (Ohkuma and Poole, 1978) and low temperature (Levy et al., 1959; Poole and Ohkuma, 1981) retard the process. It is not surprising, therefore, to find that the size of vacuoles obtained after incubation of Clone 9 hepatocytes with NH_4Cl or chloroquine at 20°C is smaller than those obtained after incubation for comparable treatment time periods at 37°C, since proton pumping and vesicle fusion are energy and, hence, temperature dependent events.

The possibility that the low temperature itself might cause vesiculation of the Golgi complex had to be considered, since previous studies on this subject have shown that this phenomenon does indeed occur

at 16°C (Griffiths et al., 1989). If this were the case, then our initial experimental question could not be answered by immunocytochemical techniques. To assess this possibility, Man6P receptors in Clone 9 hepatocytes were localized at the electron microscopic level after incubation of untreated cells at 20°C for 3.5 h. The Golgi complex did retain its characteristic cisternal morphology at the lower temperature (Fig. 2B), implying that the vacuolar IF pattern after weak base treatment truly represented endosomes loaded with Man6P receptors and not vesiculated Golgi components (Fig. 3).

Parallel immunoelectron microscopic studies were done to localize the Man6P receptor in Clone 9 hepatocytes after treatment of cells with NH_4Cl or chloroquine for 3.5 h at 37°C and at 20°C. Man6P receptors were localized to large dilated endosomes after 3.5 h of treatment with either weak base at either temperature. These findings corroborated IF data demonstrating that the "Endosomal" IF staining pattern representing, at the electron microscopic level, the presence of Man6P receptors in large dilated endosomes. Furthermore, these findings agree with the immunoelectron localization studies reported by Brown and Farquhar (1986) using lysosomotropic agents to follow Man6P receptors in Clone 9 hepatocytes.

The traffic of Man6P receptors from endosomes back to the Golgi complex was studied by loading receptors into endosomes by treatment with NH_4Cl or chloroquine. When the base is washed out of the cells, the intra-endosomal pH is re-established, thus allowing Man6P receptors to dissociate from lysosomal enzymes and recycle back to the Golgi complex. The traffic of Man6P receptors during this return trip at 20°C was also compared to recycling at 37°C. From the data compiled from this second

set of experiments, it became clear that Man6P receptor trafficking could not be disrupted at 20°C. In fact, receptors did complete the trip from endosomes back to the Golgi complex, albeit at a slower rate. Although the washout of chloroquine is more difficult to achieve because of the lipophilic nature of the base, similar results were obtained after washout at the two temperatures.

The finding that Man6P receptor traffic between endosomes and the Golgi complex is not disrupted at 20°C was surprising since so many earlier studies had demonstrated that intracellular pathways, especially in the exocytic direction, were affected at low temperature. In this context, Man6P receptor traffic may be unique. Unfortunately, our knowledge of the molecular events along intracellular transport pathways and our understanding of the precise molecular changes that occur at low temperatures is rudimentary at best.

The current findings are, however, consistent with a recent study conducted by Gabel and Foster (1987) who demonstrated that lowering the temperature of murine L-cells to 20°C led to the accumulation of newly endcytosed ß-glucuronidase in an intracellular acidic compartment. Since the progressive accumulation of endocytosed extracellular enzyme continued despite the low temperature, these investigators concluded that the Man6P receptors must continue to recycle at 20°C from the acidic compartment back to the cell surface, presumably to pick up another enzyme cargo. Their data imply that the fusion of endocytic and lysosomal vesicles is a temperature sensitive step, but that Man6P-receptor-mediated internalization of ß-glucuronidase by murine L-cells is not. Thus, the question arises: do the energy requirments of membrane fusion at the plasma membrane level differ from the energy requirements of

intracellular fusion events, since the transport of Man6P receptors presumably occurs via coated vesicles and these receptors were able to return to the cell surface despite the low temperature?

In conclusion, the findings presented here indicate that the Man6P receptor is unique in its behavior at low temperature since traffic along the outbound direction from the Golgi complex to endosomes is not disrupted in contrast to previous studies of other cell systems which investigated the traffic along the Golgi to plasmalemma pathway. Furthermore, the data presented here suggesting Man6P receptor traffic along the pathway between endosomes and the Golgi complex proceeds at 20°C agree with those results reported by Gabel and Foster (1987), indicating that recycling of Man6P receptors from the endosomes back to the plasma membrane occurs at the same low temperature. The 20°C temperature block could potentially be used as a tool to dissociate lysosomal enzyme traffic (which proceeds at 20°C) from exocytic traffic (which is disrupted at 20°C) and could become an interesting tool in the development of future studies.

Many questions remain unanswered about the molecular mechanisms of intracellular transport and the role that temperature changes play in disrupting molecular structure. The results presented here, that Man6P receptor traffic indeed proceeds at a temperature well below physiologic temperature, make us realize that our understanding of such processes is only in its infancy.

TABLES AND FIGURES

TABLE I

EFFECT OF AMMONIUM CHLORIDE ON THE DISTRIBUTION OF MAN6P RECEPTORS IN CLONE 9 HEPATOCYTES

WEAK BASE TREATMENT	GOLGI	MIXED	ENDOSOME	n; ± SD
Steady state, 37°C	100 %	0 %	0 %	3; ± 0 %
Steady state, 20°C	100 %	0 %	0 %	3; ± 0 %
1h NH_4Cl, 37°C	0 %	69 %	31 %	3; ± 2 %
1h NH_4Cl, 20°C	16 %	84 %	0 %	3; ± 5 %
2 h NH_4Cl, 37°C	0 %	44 %	56 %	3; ± 2 %
2 h NH_4Cl, 20°C	0 %	71 %	29 %	2; ± 6 %
3.5h NH_4Cl, 37°C	0 %	7 %	93 %	3; ± 1 %
3.5h NH_4Cl, 20°C	0 %	4 %	96 %	2; ± 4 %

Clone 9 hepatocytes were treated with NH_4Cl for 1 h, 2 h, and 3.5 h. The number of cells with a "Golgi," "Mixed," or "Endosomal" IF staining pattern was scored after treatment for each time period in each experiment. The results were compiled from all experiments, averaged, and standard deviations (SD) calculated.

TABLE II

EFFECT OF CHLOROQUINE ON THE DISTRIBUTION OF MAN6P RECEPTORS IN CLONE 9 HEPATOCYTES

WEAK BASE TREATMENT	GOLGI	MIXED	ENDOSOME	n; ± SD
Steady state, 37°C	100 %	0 %	0 %	3; ± 0 %
Steady state, 20°C	100%	0 %	0 %	3; ± 0 %
1 h chloroquine, 37°C	0 %	38 %	62 %	2; ± 13 %
1 h chloroquine, 20°C	26 %	74 %	0 %	2; ± 4 %
2 h chloroquine, 37°C	0 %	7 %	93 %	3; ± 2 %
2 h chloroquine, 20°C	0 %	26 %	74 %	2; ± 4 %
3.5 h chloroquine, 37°C	0 %	4 %	96 %	3; ± 2 %
3.5 h chloroquine, 20°C	0 %	21 %	79 %	3; ± 1 %

Clone 9 hepatocytes were treated with chloroquine for 1 h, 2 h, and 3.5 h. The number of cells with a "Golgi," "Mixed," or "Endosomal" IF staining pattern was scored after treatment for each time period in each experiment. The results were compiled from all experiments, averaged, and standard deviations (SD) calculated.

TABLE III

EFFECT OF AMMONIUM CHLORIDE REMOVAL ON THE RECYCLING OF MAN6P RECEPTORS BACK TO THE GOLGI COMPLEX IN CLONE 9 HEPATOCYTES

WEAK BASE TREATMENT	ENDOSOME	MIXED	GOLGI	n; ± SD
3.5h NH_4Cl, 37°C	93 %	4 %	0 %	4; ± 1 %
3.5h NH_4Cl, 20°C	91 %	9 %	0 %	3; ± 1 %
3.5h NH_4Cl, 37°C + 30 min washout, 37°C	0 %	18 %	82 %	4; ± 6 %
3.5h NH_4Cl, 37°C + 30 min washout, 20°C	0 %	73 %	27 %	3; ± 9 %
3.5h NH_4Cl, 37°C + 1h washout, 37°C	0 %	5 %	95 %	3; ± 6 %
3.5h NH_4Cl, 37°C + 1h washout, 20°C	0 %	72 %	28 %	3; ± 8 %
3.5h NH_4Cl, 37°C + 2h washout, 37°C	0 %	1 %	99 %	3; ± 2 %
3.5h NH_4Cl, 20°C + 2h washout, 20°C	0 %	29 %	71 %	3; ± 11 %

Clone 9 hepatocytes were treated with NH_4Cl for 3.5 h to load the bulk of the Man6P receptors into endosomes. Then cells were washed in NH_4Cl-free medium for 30 min, 1 h, and 2 h to follow receptors as they recycled back to the Golgi complex. The number of cells with a "Golgi," "Mixed," and "Endosomal" IF staining pattern after each period of washout was quantitated. Results were averaged from three or four experiments and standard deviations (SD) calculated.

TABLE IV

EFFECT OF CHLOROQUINE REMOVAL ON THE RECYCLING OF MAN6P RECEPTORS BACK TO THE GOLGI COMPLEX IN CLONE 9 HEPATOCYTES

WEAK BASE TREATMENT	ENDOSOME	MIXED	GOLGI	n; ± SD
3.5h chloroquine, 37°C	97 %	3 %	0 %	3; ± 3 %
3.5h chloroquine, 20°C	79 %	21 %	0 %	3; ± 1 %
3.5h chloroquine, 37°C + 1h washout, 37°C	83 %	17 %	0 %	3; ± 3 %
3.5h chloroquine, 37°C + 1h washout, 20°C	87 %	13 %	0 %	2; ± 4 %
3.5h chloroquine, 37°C + 2h washout, 37°C	0 %	68 %	32 %	3; ± 5 %
3.5h chloroquine, 37°C + 2h washout, 20°C	71 %	29 %	0 %	3; ± 1 %

Clone 9 hepatocytes were treated with chloroquine for 3.5 h to load the bulk of the Man6P receptors into endosomes. Then cells were washed in chloroquine-free medium for 30 min, 1 h, and 2 h to follow receptors as they recycled back to the Golgi complex. The number of cells with a "Golgi," "Mixed," and "Endosomal" IF staining pattern after each period of washout was quantitated. Results were averaged from three experiments and standard deviations (SD) calculated.

Figure 1

Immunofluorescent localization of Man6P receptors in Clone 9 hepatocytes at steady state. Pairs of micrographs are shown of the same field by fluorescence (a, b) or phase-contrast (a', b') microscopy. Control (untreated) cells (a, a') at steady state at 37°C and at 20°C (b, b'). There is clearly a juxtanuclear "Golgi" staining pattern (G) in control (untreated) cells at steady state at 37°C and at 20°C. Fig. 1, x 1,800.

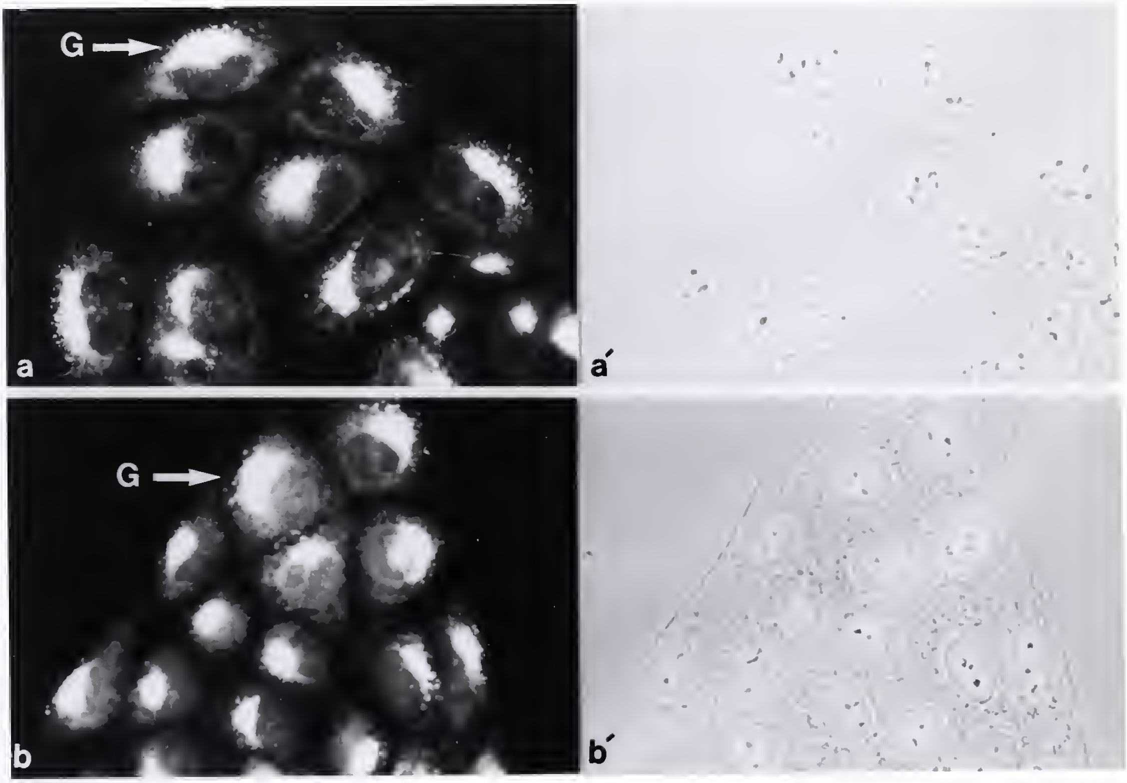

G
a
a´
G
b
b´

Figure 2

Immunoperoxidase localization of Man6P receptors at steady state in control (untreated) Clone 9 hepatocytes.

(A) At 37°C, Man6P receptors are localized to single stacked Golgi cisternae (Gc), in small vesicles (ve) in the Golgi region or in occasional small endosomes (en). Little reaction product is detectable in multivesicular bodies (mv). Fig. 2A, x 24,000.

(B) At 20°C, the peroxidase localization of Man6P receptors is the same as at 37°C. Even at the lower temperature, the Golgi complex retains its characteristic cisternal morphology. Fig. 2B, x 24,000

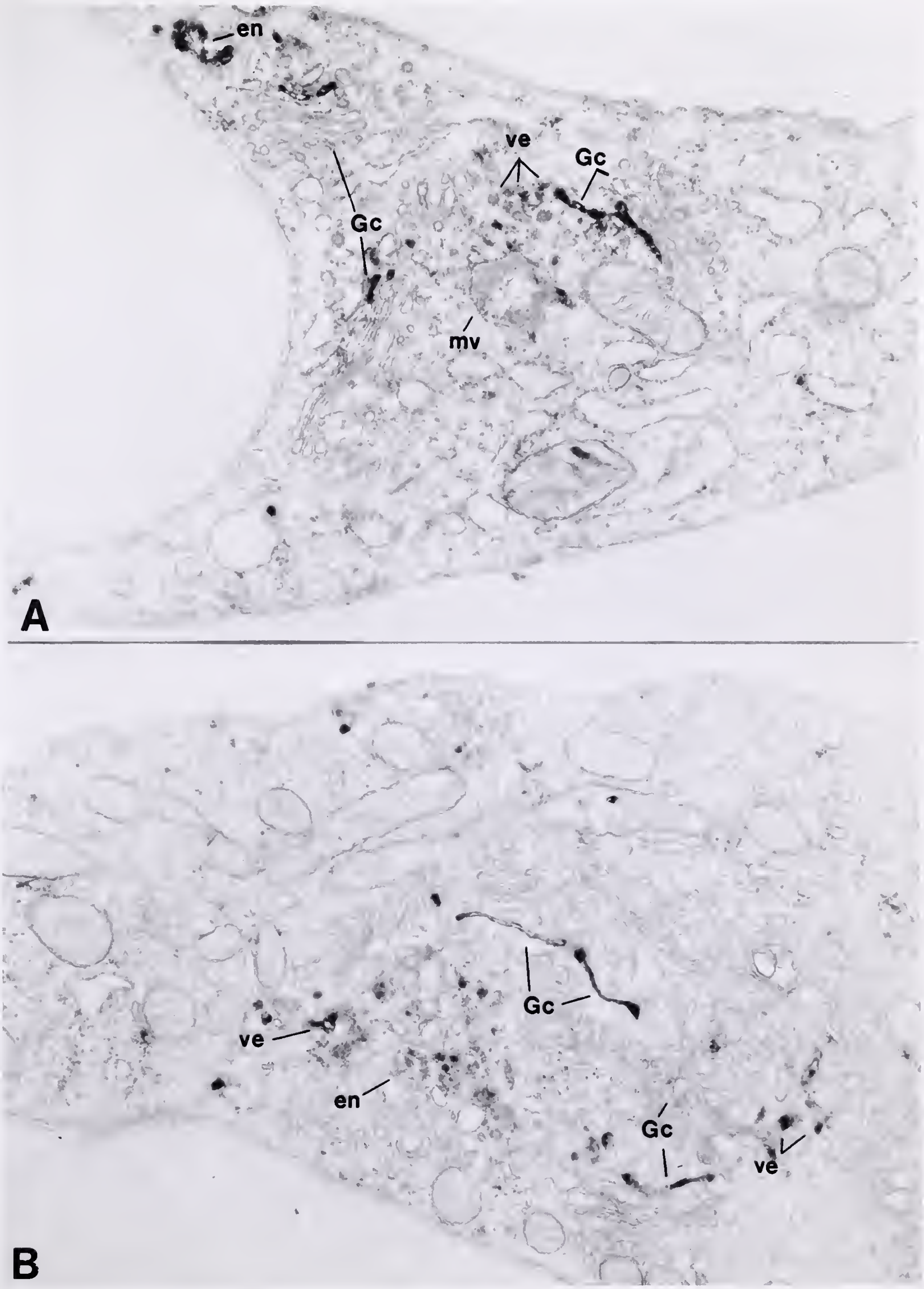

en
ve
Gc
Gc
mv
A
Gc
ve
en
Gc
ve
B

Figure 3

Immunofluorescent localization of Man6P receptors after the addition of chloroquine for 3.5 h at 37°C (a, a') versus 20° (b, b'). By phase contrast microscopy, the number and size of phase-lucent vacuoles (endosomes) increase after weak base treatment. A change is observed in the distribution of the Man6P receptors from the juxtanuclear "Golgi" staining pattern in controls to a vacuolar (endosomal) staining pattern (E) after chloroquine treatment. It is clear by phase contrast or immunofluorescence examination that endosomes (e) are not as dilated after treatment at 20°C as they are after treatment at 37°C (a' versus b'). Fig. 3, x 1,800.

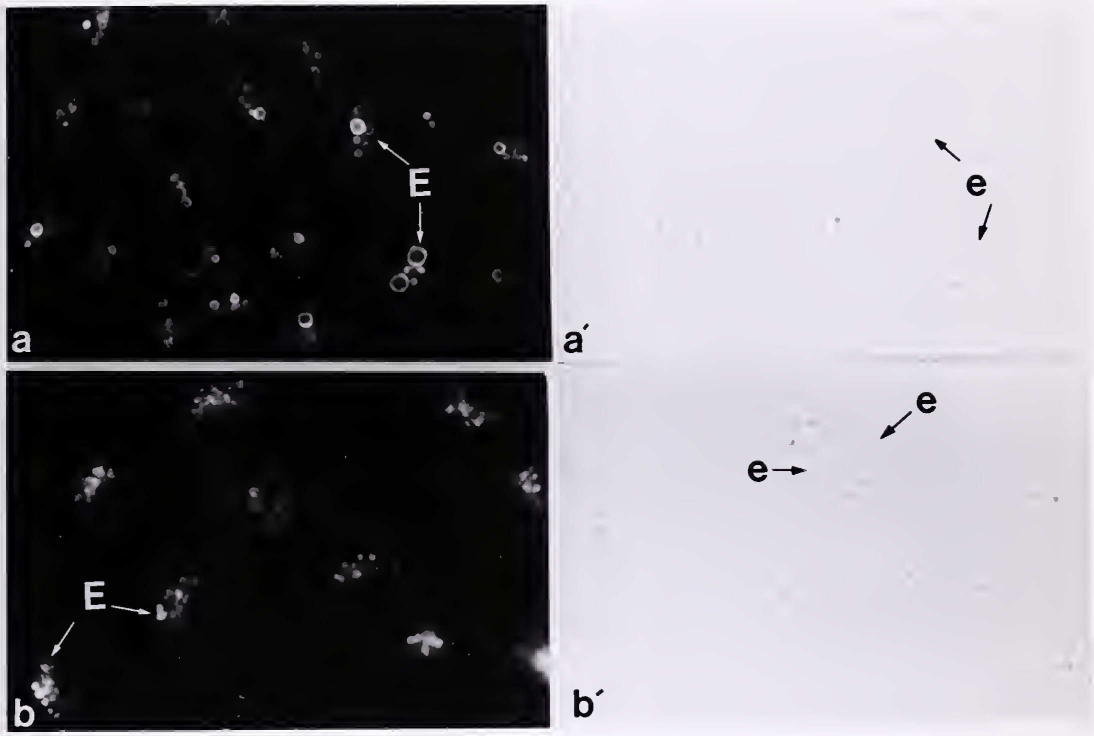

a
E
a′
e
b
E
b′
e
e

Figure 4

Redistribution of Man6P receptors after the addition of NH_4Cl at 37°C (a, b, c) and at 20°C (d, e, f).

(a) after 1 h of NH_4Cl treatment at 37°C, most cells have a "Mixed Golgi-Endosome" (M) staining pattern.

(b) after 2 h of NH_4Cl treatment at 37°C, most cells already have an 'Endosomal' (E) staining pattern.

(c) after 3.5 h of NH_4Cl treatment at 37°C, nearly all cells have an "Endosomal" staining pattern (E).

(d) after 1 h of NH_4Cl treatment at 20°C, most cells have a 'Mixed' (M) staining pattern while a significant proportion have the 'Golgi' pattern characteristic of controls.

(e) after 2 h of NH_4Cl treatment at 20°C, most cells still have a "Mixed Golgi-Endosome" (M) staining pattern.

(f) after 3.5 h of treatment at 20°C, most cells have an "Endosomal" staining pattern (E). Although dilated endosomes can be seen on fluorescence, it is clear that endosomes do not become as dilated when cells are treated at 20°C as they do at 37°C Fig. 4, x 1,600.

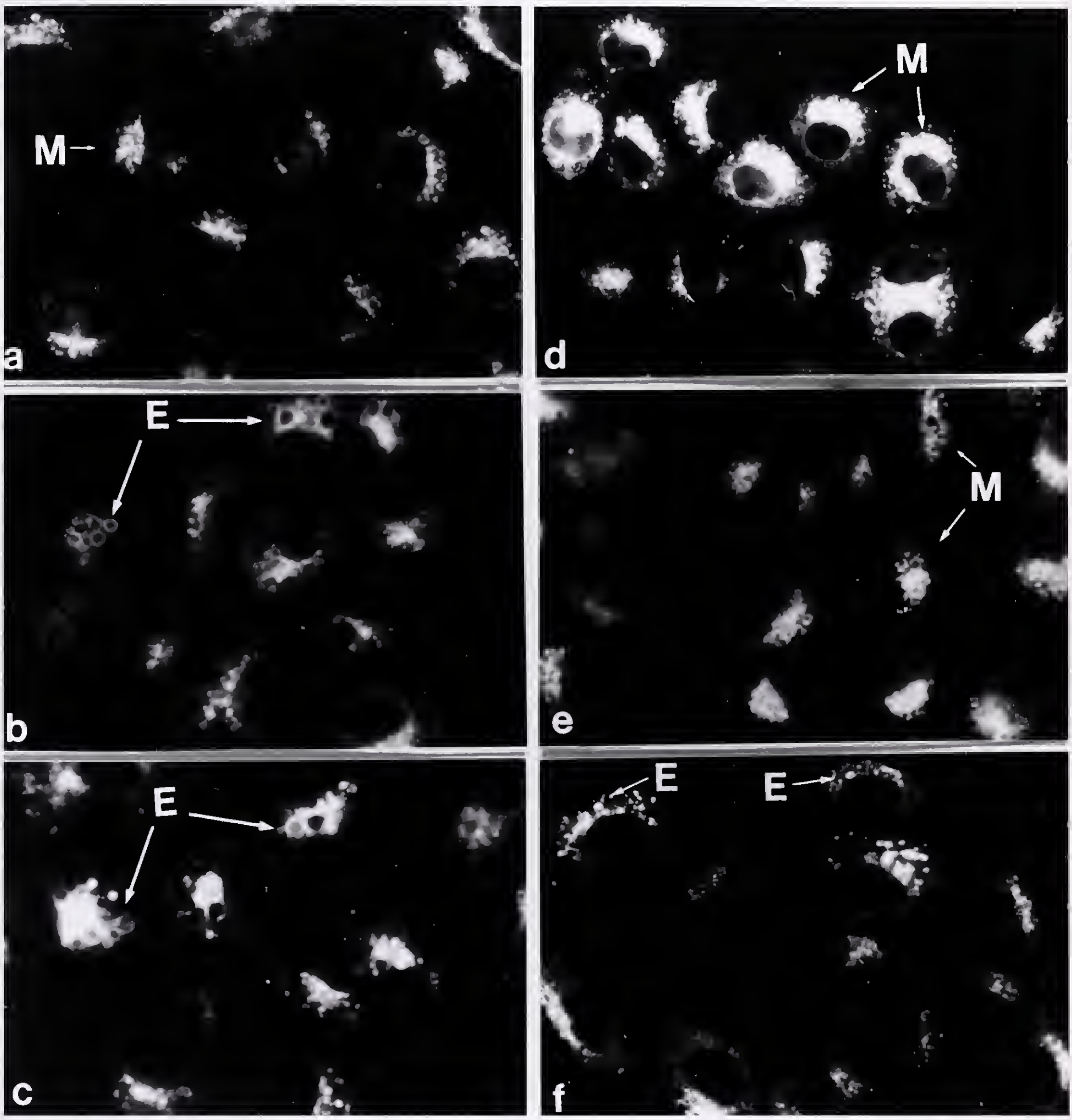

M
a
M
M
d
E
E
b
M
e
E
E
c
E
E
f

Figure 5

Immunoperoxidase localization of Man6P receptors in Clone 9 hepatocytes after chloroquine treatment at 37°C versus 20°C. After incubation of Clone 9 cells in MEM containing 25 µM chloroquine for 3.5 h at 37°C (A) and at 20°C (B), Man6P receptors are found predominately in large dilated endosomes (en) and are depleted from the Golgi cisternae (Gc). Fig. 5A, x 21,000 and Fig. 5B, x 24,000.

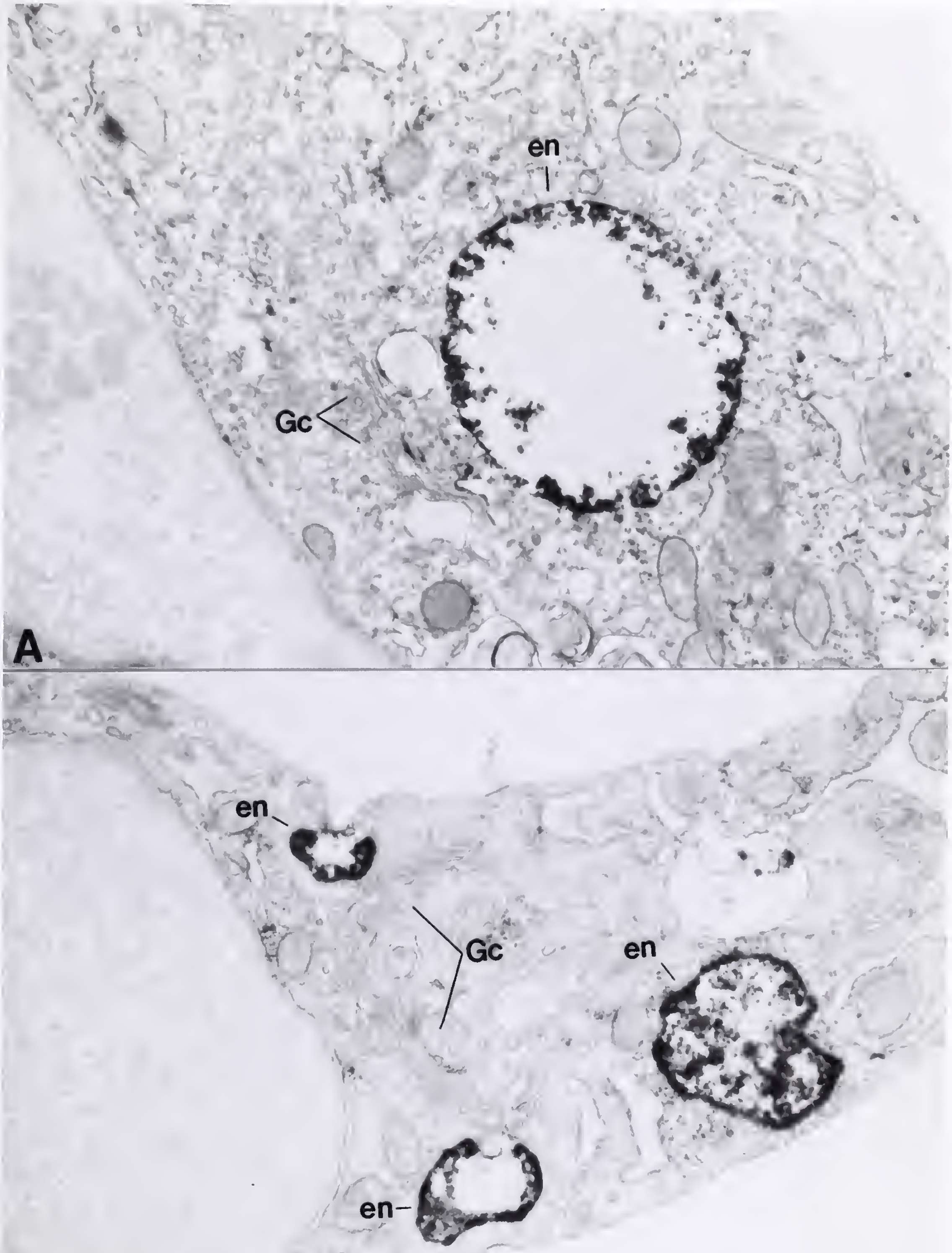

en
Gc
A
en
Gc
en
en
B

Figure 6

Localization of Man6P receptors in Clone 9 hepatocytes after the removal of NH_4Cl. Following NH_4Cl treatment of cells for 3.5 h at 37°C (a, a'), endosomes (E) become dilated. After treatment of Clone 9 hepatocytes with weak base (NH4Cl or chloroquine) for 3.5 h, and then washing out the weak base at 37°C for 2 h in base-free MEM, it can be demonstrated by fluorescence (b) and phase contrast microscopy (b') that cells return to their pre-treatment morphology with Man6P receptors localized in the juxtanuclear Golgi region (G). This pattern is characteristic of cells washed with NH_4Cl-free or chloroquine-free media. Fig. 6, x 1,600.

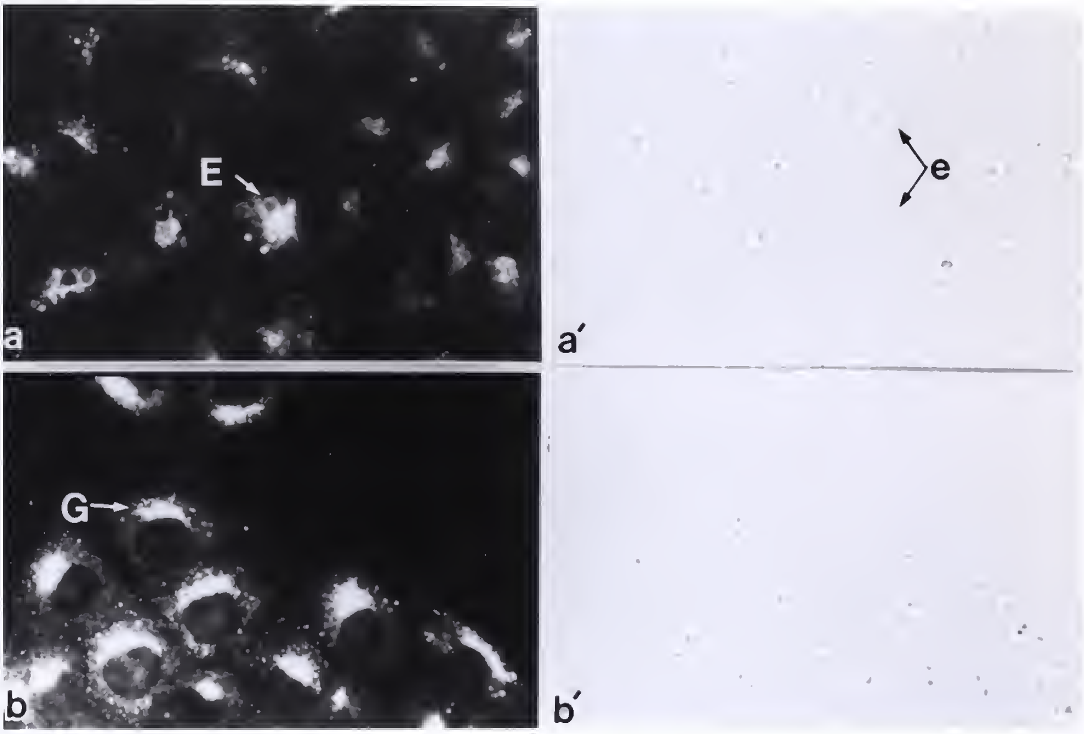

E
e
G
a
a′
b
b′

Figure 7

Redistribution of Man6P receptors after treating Clone 9 hepatocytes with chloroquine for 3.5 h at 37°C (a) and then incubating cells in chloroquine-free medium at 37°C (b, c, d) versus 20°C (e, f, g). Cells were incubated in chloroquine-free medium for 30 min, 1 h and 2 h at 37°C (b, c, d) and at 20°C (e, f, g).

(a) after treatment for 3.5 h at 37°C, virtually all Man6P receptors become localized to large dilated endosomes (E).

(b) after washout of chloroquine for 30 min at 37°C, cells begin to manifest a "Mixed" IF pattern indicating movement of Man6P receptors from endosomes back to the Golgi complex.

(c) after washout of chloroquine for 1 h, the majority of cells have a “Mixed" (M) IF pattern.

(d) after washout of chloroquine for 2 h, a number of cells have the “Golgi” IF pattern characteristic of controls indicating that receptors have completed their transit from endosomes back to the Golgi complex.

(e) after washout of chloroquine for 30 min at 20°C, cells still retain an "Endosomal" IF pattern.

(f) after washout of chloroquine for 1 h at 20°C, most cells still have an "Endosomal" IF pattern.

(g) after washout of chloroquine for 2 h at 20°C, a fraction of cells begin to manifest a "Mixed" IF signal indicating some movement of receptors back to the Golgi complex but no pure “Golgi” signal is yet visible. Fig. 7, x 1,700.

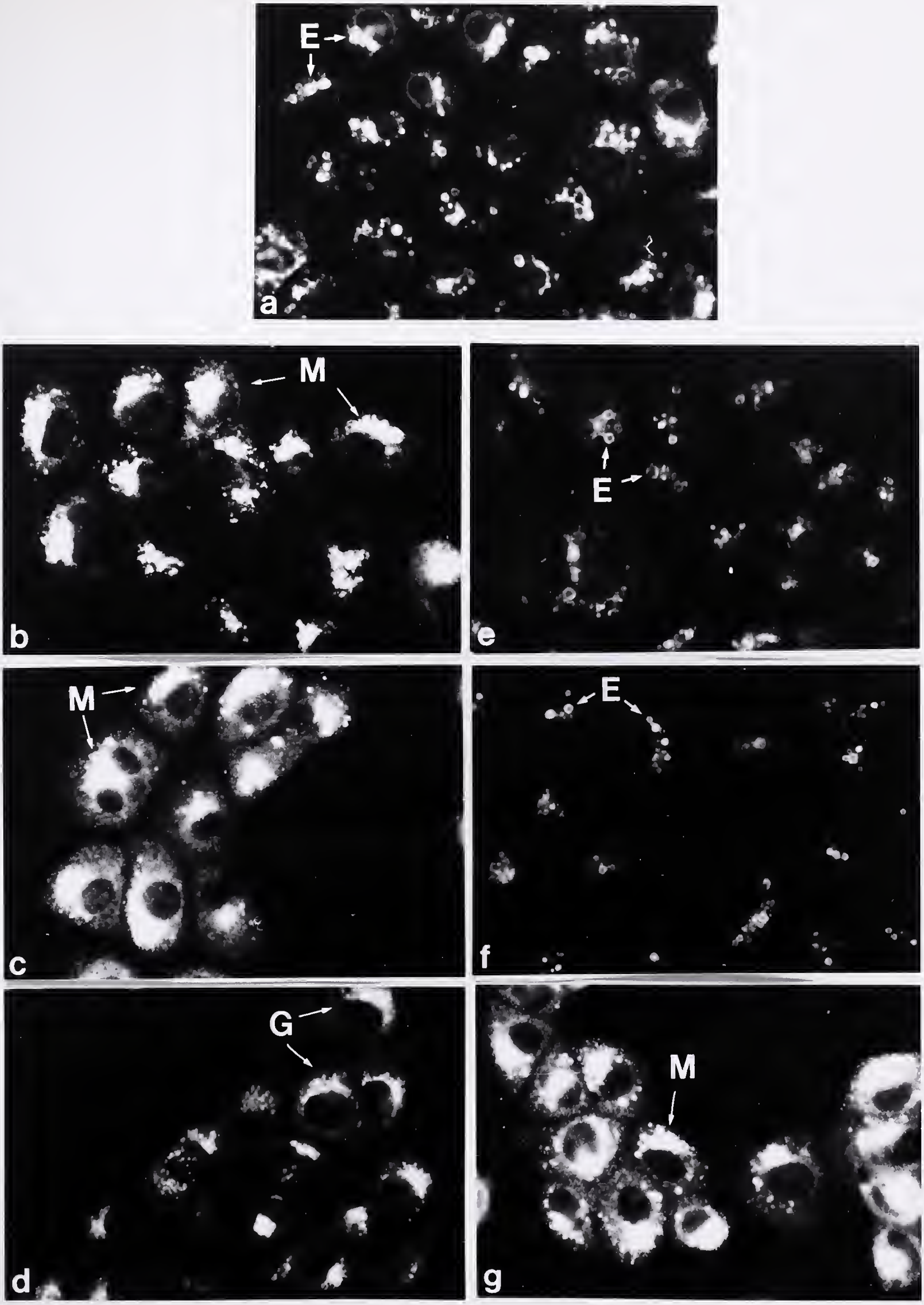

E
E
a
M
b
E
e
M
c
E
f
G
d
M
g

Figure 8

Immunoperoxidase localization of Man6P receptors after washout for 30 min at 37°C (A) and at 20°C (B). Clone 9 hepatocytes were pretreated with chloroquine for 3.5 h at 37°C before the washout period.

(A) After transfer of cells to chloroquine-free MEM for 30 min at 37°C, it is clear Man6P receptors are predominately found in large dilated endosomes (en) although some receptors can already be detected in the Golgi cisternae (Gc) as well. Fig. 8A, x 24,000.

(B) After transfer of cells to chloroquine-free MEM for 30 min at 20°C, the bulk of immunodetectable Man6P receptors remains trapped in large dilated endosomes. Fig. 8B, x 24,000.

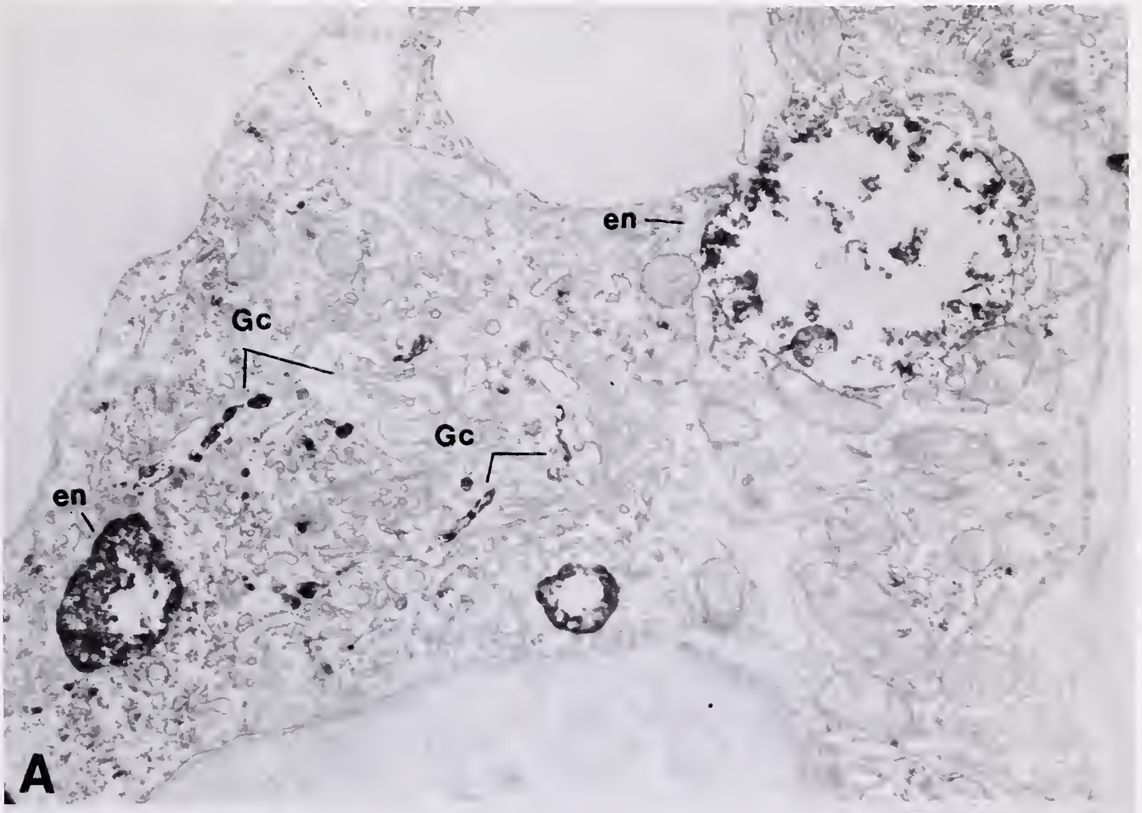

en
Gc
Gc
en
A
en
Gc
B

Figure 9

Immunoperoxidase localization of Man6P receptors after washout of chloroquine for 1 h at 37°C (A) and at 20°C (B). Clone 9 hepatocytes were pretreated with chloroquine for 3.5 h at 37°C before the washout period.

(A) After transfer to chloroquine-free MEM for 1 h at 37°C, it is clear that the Golgi cisternae (Gc) are strongly stained with diaminobenzidine reaction product indicating that Man6P receptors have recycled to the Golgi from endosomes (en). The finding of immunodetectable receptor in both endosomes and the Golgi complex represents what is described as a "Mixed" signal at the IF level, indicating staining of both endosomes and the Golgi complex. Fig. 9A, x 24,000.

(B) After 1 h in chloroquine-free MEM at 20°C, one sees dilated endosomes (en) and there is little if any immunodetectable receptor in the Golgi cisternae. This represents what is referred to as a predominately "Endosomal" signal at the IF level. Fig. 9B, x 24,000.

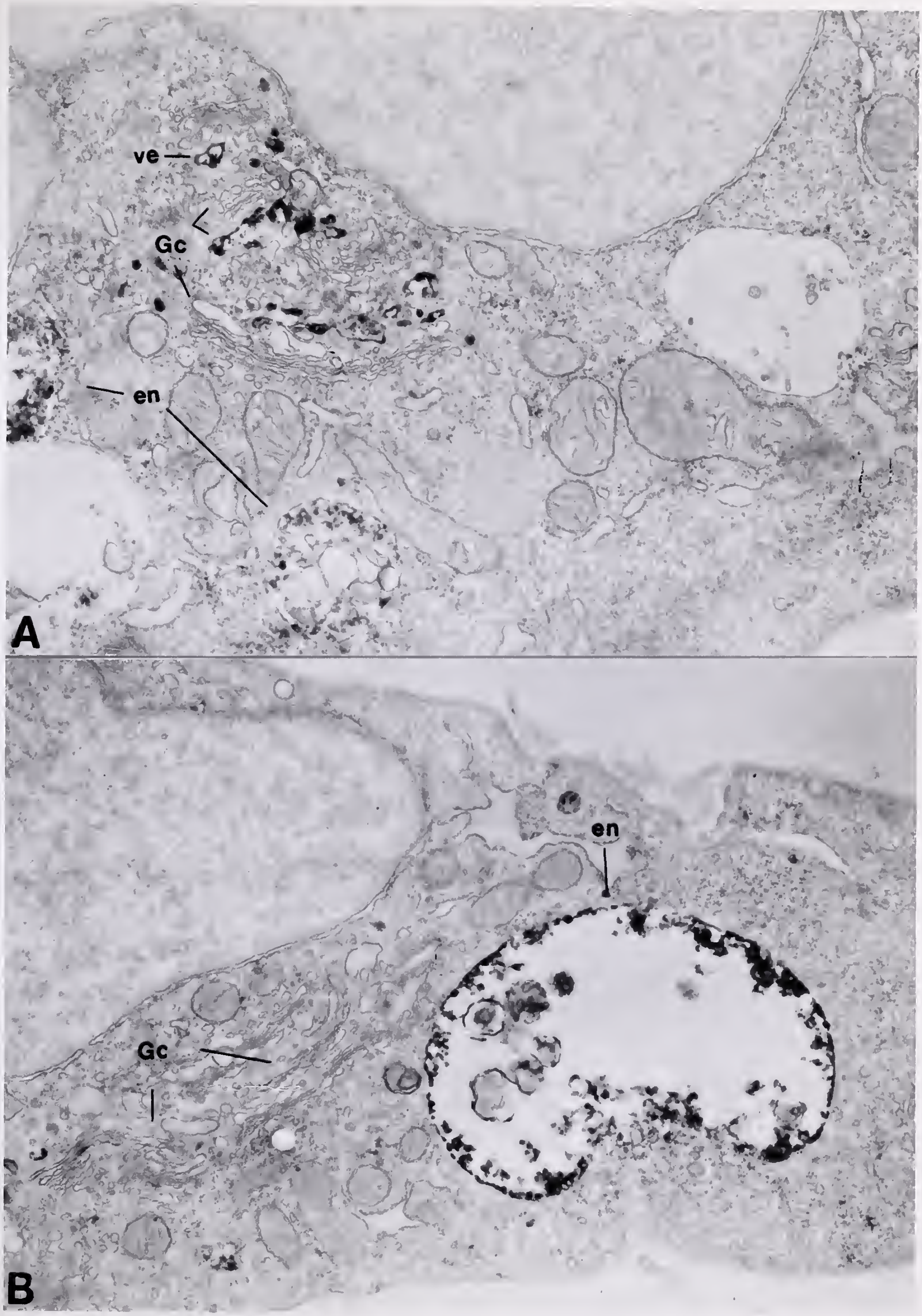

ve
Gc
en
A
en
Gc
B

Figure 10

Immunoperoxidase labeling of Clone 9 hepatocytes after washout of chloroquine for 2 h at 37°C (A) and at 20°C (B). Clone 9 hepatocytes were pretreated with chloroquine for 3.5 h at 37°C before the washout period.

(A) some Man6P receptors have recycled back to the Golgi complex after 2 h in chloroquine-free medium at 37°C, but a significant proportion of immunodetectable receptor still remain in endosomes (en). Fig. 10A, x 24,000.

(B) even after 2 h of washout at 20°C no receptors are detectable in the Golgi complex (Gc). This immunoperoxidase localization confirms the IF results reported above; namely, that there are still Man6P receptors present in both endosomes and the Golgi complex after 2 h of washout at 37°C representing a "Mixed" signal at the IF level. Receptors remain predominately in the endosomes after the 2 h washout at 20°C which represents what is referred to here as a pure "Endosomal" signal at the IF level. Fig. 10B, x 24,000.

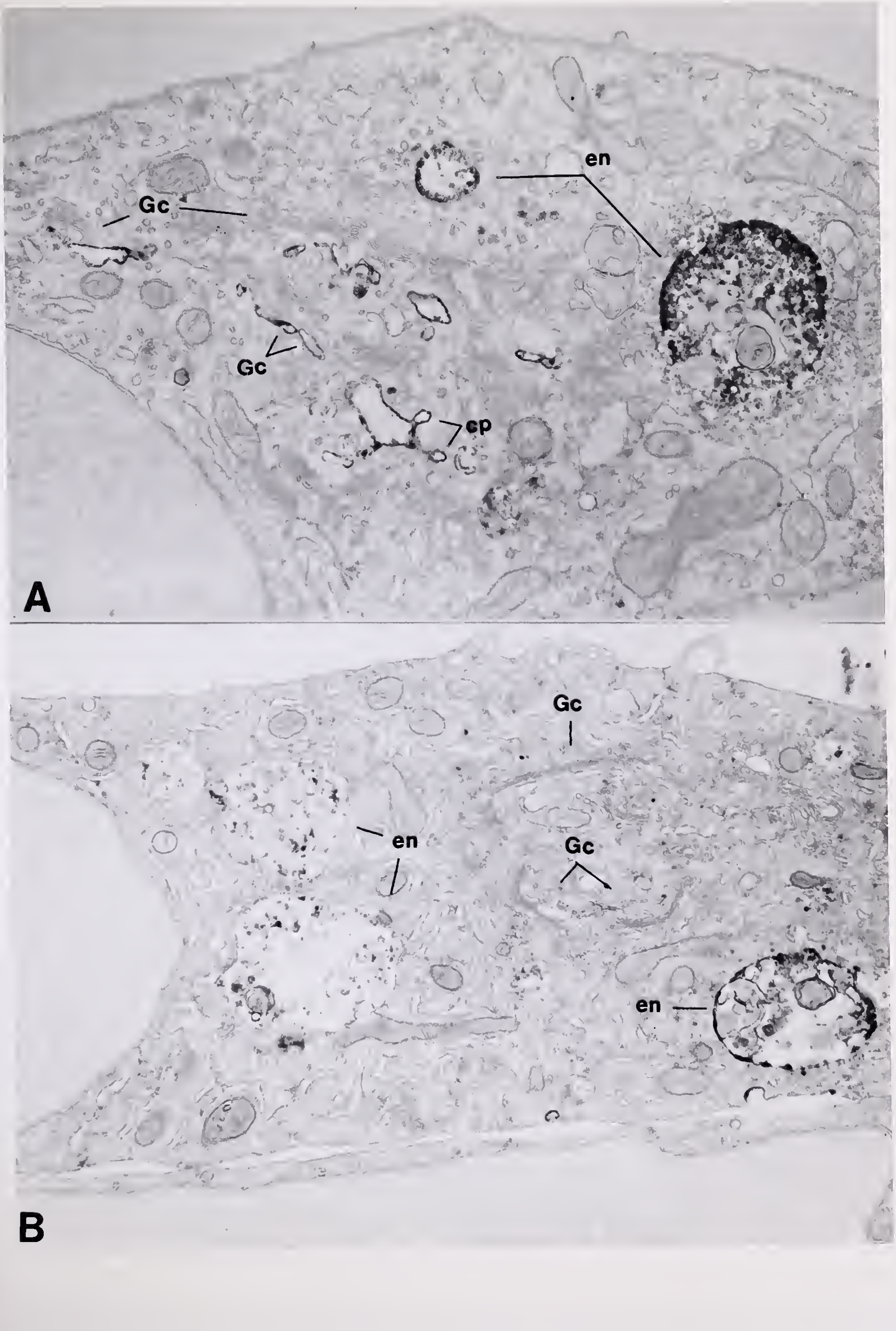
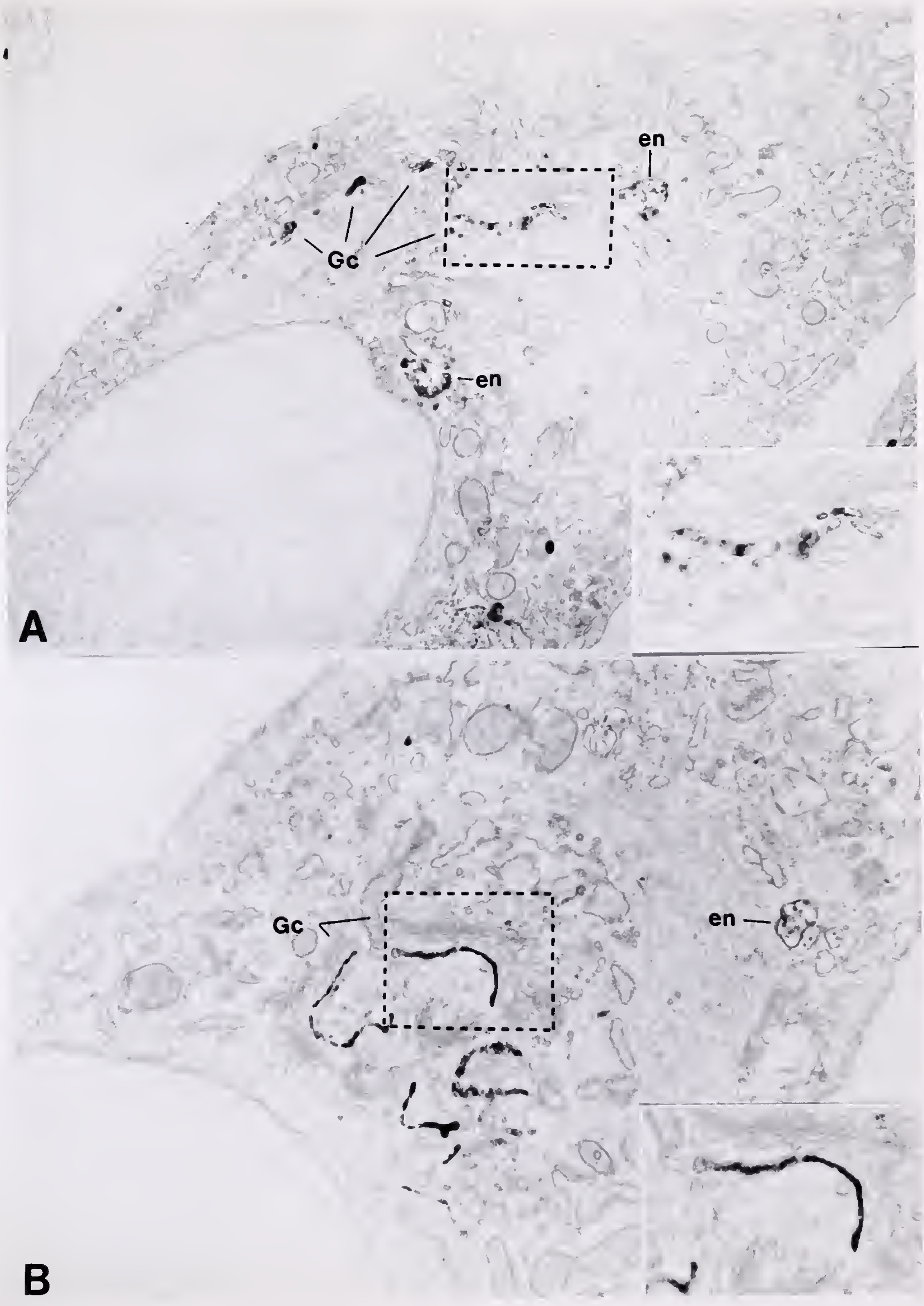

en
Gc
Gc
cp
A
Gc
en
Gc
en
B

Figure 11

Immunoperoxidase localization of Man6P receptors after washout of chloroquine (A) and NH_4Cl (B) at 37°C. Clone 9 hepatocytes were pretreated with weak base for 3.5 h at 37°C before the washout period.

(A) A fraction of cells (32%) have a pure “Golgi” IF signal after 2 h washout of chloroquine at 37°C represented here by Man6P receptor localization predominately in the Golgi complex (Gc) with very little endosomal labeling (en). Fig. 11A, x 24,000.

(B) In the case NH_4Cl washout, an even greater proportion of cells (99%) have a pure "Golgi" signal at the IF level represented here as peroxidase reaction product localized to the cisternae of the Golgi complex without dilated endosomes (en) present. Fig. 11B, x 25,000.


en
Gc
en
A
Gc
en
B

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